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BASIC LABORATORY PROCEDURES IN CLINICAL BACTERIOLOGY

2nd edition

World Health Organization
Geneva

Basic laboratory procedures in clinical bacteriology

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Basic laboratory procedures in clinical bacteriology

Second edition

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Contents

Preface viii

Introduction 1

Quality assurance in bacteriology 2

Introduction 2
Definitions 2
Internal quality control 6
External quality assessment 16

PART I
Bacteriological investigations 19

Blood 20

Introduction 20
When and where bacteraemia may occur 20
Blood collection 20
Blood-culture media 22
Processing of blood cultures 23

Cerebrospinal fluid 25

Introduction 25
Collection and transportation of specimens 25
Macroscopic inspection 26
Microscopic examination 26
Preliminary identification 28
Susceptibility testing 29

Urine 30

Introduction 30
Specimen collection 30
Culture and interpretation 32
Interpretation of quantitative urine culture results 34
Identification 35
Susceptibility tests 36

Stool 37

Introduction 37
Etiological agents and clinical features 37
Appropriate use of laboratory resources 39
Collection and transport of stool specimens 40
Visual examination of stool specimens 41
Enrichment and inoculation of stool specimens 41
Media for enteric pathogens 42
Primary isolation 42
Preliminary identification of isolates 44

Final microbiological identification 50
Serological identification 54

Upper respiratory tract infections 60

Introduction 60
Normal flora of the pharynx 60
Bacterial agents of pharyngitis 61
Collection and dispatch of specimens 62
Direct microscopy 62
Culture and identification 63
Susceptibility testing 65

Lower respiratory tract infections 66

Introduction 66
The most common infections 66
Collection of sputum specimens 68
Processing of sputum in the laboratory (for non-tuberculous infections) 68
Culture for *Mycobacterium tuberculosis* 72
Interpretation of cultures for *M. tuberculosis* 74
General note on safety 74

Sexually transmitted diseases 76

Introduction 76
Urethritis in men 77
Genital specimens from women 79
Specimens from genital ulcers 82

Purulent exudates, wounds and abscesses 86

Introduction 86
Commonly encountered clinical conditions and the most frequent etiological agents 86
Collection and transportation of specimens 89
Macroscopic evaluation 90
Microscopic examination 91
Culture 92
Identification 93
Susceptibility testing 97

Anaerobic bacteriology 98

Introduction 98
Description of bacteria in relation to oxygen requirement 98
Bacteriology 98

Antimicrobial susceptibility testing 103

Introduction 103
General principles of antimicrobial susceptibility testing 103
Clinical definition of terms “resistant” and “susceptible”: the three category system 104
Indications for routine susceptibility tests 106

Choice of drugs for routine susceptibility tests in the clinical laboratory 107
The modified Kirby–Bauer method 109
Direct versus indirect susceptibility tests 117
Technical factors influencing the size of the zone in the disc-diffusion method 118
Quality control 120

Serological tests 122

Introduction 122
Quality control measures 122
Serological reactions 125
Serological tests for syphilis 126
Febrile agglutinins tests 133
Antistreptolysin O test 135
Bacterial antigen tests 137

PART II
Essential media and reagents 141

Introduction 142

Pathogens, media and diagnostic reagents 143

Blood 144
Cerebrospinal fluid 144
Urine 145
Stool 146
Upper respiratory tract 147
Lower respiratory tract 148
Urogenital specimens for exclusion of sexually transmitted diseases 149
Pus and exudates 149
List of recommended media and diagnostic reagents for the intermediate microbiological laboratory 150

Selected further reading 154

Index 155

Introduction

Communicable diseases continue to account for an unduly high proportion of the health budgets of developing countries. According to *The world health report*,[1] acute diarrhoea is responsible for as many as 2.2 million deaths annually. Acute respiratory infections (primarily pneumonia) are another important cause of death, resulting in an estimated 4 million deaths each year. Analysis of data on lung aspirates appears to indicate that, in developing countries, bacteria such as *Haemophilus influenzae* and *Streptococcus pneumoniae*, rather than viruses, are the predominant pathogens in childhood pneumonia. β-Lactamase-producing *H. influenzae* and *S. pneumoniae* with decreased sensitivity to benzylpenicillin have appeared in different parts of the world, making the surveillance of these pathogens increasingly important.

Sexually transmitted diseases are on the increase. There are still threats of epidemics and pandemics of viral or bacterial origin, made more likely by inadequate epidemiological surveillance and deficient preventive measures. To prevent and control the main bacterial diseases, there is a need to develop simple tools for use in epidemiological surveillance and disease monitoring, as well as simplified and reliable diagnostic techniques.

To meet the challenge that this situation represents, the health laboratory services must be based on a network of laboratories carrying out microbiological diagnostic work for health centres, hospital doctors, and epidemiologists. The complexity of the work will increase from the peripheral to the intermediate and central laboratories. Only in this way will it be possible to gather, quickly enough, sufficient relevant information to improve surveillance, and permit the early recognition of epidemics or unusual infections and the development, application, and evaluation of specific intervention measures.

[1] *The world health report 2000*. Geneva, World Health Organization, 2000.

Quality assurance in bacteriology

Introduction

Quality assurance programmes are an efficient way of maintaining the standards of performance of diagnostic laboratories, and of upgrading those standards where necessary. In microbiology, quality goes beyond technical perfection to take into account the speed, cost, and usefulness or clinical relevance of the test. Laboratory tests in general are expensive and, with progress in medicine, they tend to use up an increasing proportion of the health budget.

Definitions

To be of good quality, a diagnostic test must be clinically relevant, i.e. it must help in the prevention or treatment of disease. Other measures of quality in a diagnostic test are:

- *Reliability*: Is the result correct?
- *Reproducibility*: Is the same result obtained when the test is repeated?
- *Speed*: Is the test rapid enough to be of use to the doctor in prescribing treatment?
- *Cost–benefit ratio*: Is the cost of the test reasonable in relation to the benefit to the patient and the community?

Factors that affect the reliability and reproducibility of laboratory results

Sources of error may include the following:

- *Personnel*. The performance of the laboratory worker or technician is directly related to the quality of education and training received, the person's experience, and the conditions of employment.
- *Environmental factors*. Inadequate working space, lighting, or ventilation, extreme temperatures, excessive noise levels, or unsafe working conditions may affect results.
- *Specimens*. The method and time of sampling and the source of the specimen are often outside the direct control of the laboratory, but have a direct bearing on the ability of the laboratory to achieve reliable results. Other factors that the laboratory can control and that affect quality are the transport, identification, storage, and preparation (processing) of specimens. The laboratory therefore has a role in educating those taking and transporting specimens. Written instructions should be made available and regularly reviewed with the clinical and nursing staff.
- *Laboratory materials*. The quality of reagents, chemicals, glassware, stains, culture media, and laboratory animals all influence the reliability of test results.
- *Test method*. Some methods are more reliable than others.
- *Equipment*. Lack of equipment or the use of substandard or poorly maintained instruments will give unreliable results.
- *Examination and reading*. Hurried reading of results, or failure to examine a sufficient number of microscope fields, can cause errors.
- *Reporting*. Transcription errors, or incomplete reports, cause problems.

Quality of interpretation of test results

Interpretation is of particular importance in microbiology. At each stage in the examination of a specimen, the results should be interpreted in order to select the optimum test, in terms of speed and reliability, for the next stage of the examination.

Quality assurance in the microbiology laboratory

Quality assurance is the sum of all those activities in which the laboratory is engaged to ensure that test results are of good quality. It must be:

— *comprehensive*: to cover every step in the cycle from collecting the specimen to sending the final report to the doctor (Fig. 1);
— *rational*: to concentrate on the most critical steps in the cycle;
— *regular*: to provide continuous monitoring of test procedures;
— *frequent*: to detect and correct errors as they occur.

GOOD-QUALITY LABORATORY SERVICES MEAN GOOD-QUALITY MEDICINE

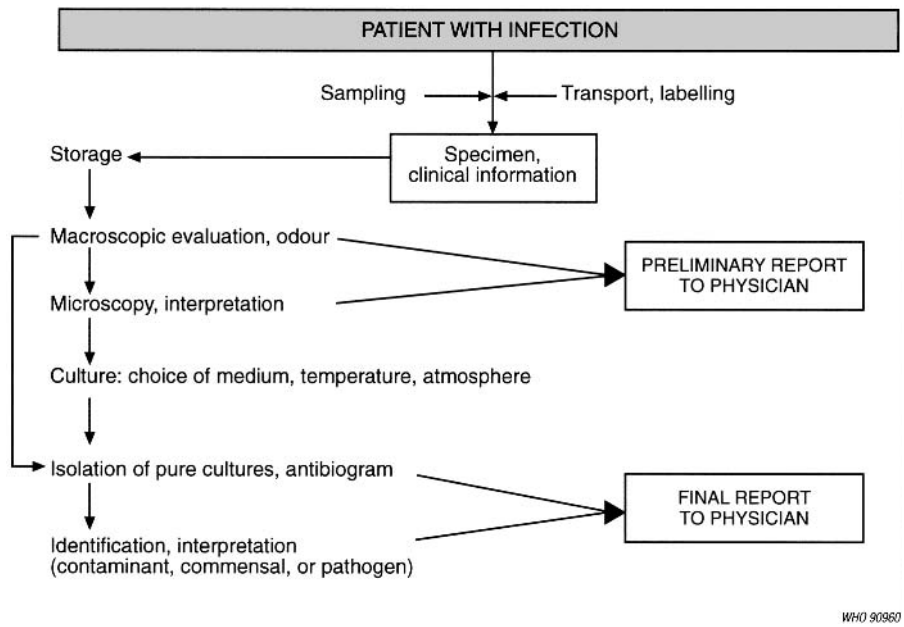
Quality assurance helps to ensure that expensive tests are used as economically as possible; it also determines whether new tests are valid or worthless, improves the performance of clinical and public health laboratories, and may help to make the results obtained in different laboratories comparable.

Types of quality assurance

There are two types of quality assurance: internal and external.

- *Internal*. This is called QUALITY CONTROL. Each laboratory has a programme to check the quality of its own tests.

Fig. 1. Steps in laboratory investigation of an infected patient



Internal quality control involves, ideally:

— *continuous monitoring* of test quality;
— *comprehensive checking* of all steps, from collecting the specimen (whenever possible) to sending the final report.

Laboratories have an ethical responsibility to the patient to produce accurate, meaningful results.

INTERNAL QUALITY CONTROL IS ABSOLUTELY ESSENTIAL FOR GOOD OPERATING PROCEDURE

- *External*. This is called QUALITY ASSESSMENT. Laboratory performance is controlled by an external agency. In some countries, participation is mandatory (regulated by the government) and required for licensure.

External quality assessment involves:

— *periodic monitoring* of test quality;
— *spot checking* of identification tests, and sometimes of isolation techniques.

Quality criteria in microbiology

Clinical relevance

An important criterion of quality for a microbiological test is how much it contributes to the prevention or cure of infectious diseases; this is called its clinical relevance. Clinical relevance can only be ensured when there is good communication between the clinician and the laboratory.

To illustrate clinical relevance, here are some examples:

1. If a few colonies of Gram-negative rods are isolated from the sputum or throat swab of a hospitalized patient, further identification and an antibiogram are of no clinical relevance, since neither procedure will have any effect on treatment of the patient.

2. If *Streptococcus pyogenes* is isolated, a full antibiogram has no clinical relevance, since benzylpenicillin is the drug of choice, and this is always active in vitro.

3. If *Escherichia coli* is isolated from a sporadic case of non-bloody diarrhoea, identification of the serotype is of no clinical relevance, since there is no clearly established correlation between serotype and pathogenicity.

4. If a Gram-stained smear shows "mixed anaerobic flora", routine identification of the anaerobes is of no clinical relevance. It would be costly in time and materials, and would not affect treatment of the patient.

5. If a yeast is isolated from a respiratory tract specimen, an identification test for *Cryptococcus* should be done. Further identification tests have no clinical relevance, since they would have no effect on patient management.

In summary, a test of good quality is one that is accurate and gives useful results for the prevention or cure of infection. It is not necessary to isolate and identify all the different types of organism in the sample.

Reliability

For tests that give quantitative results, reliability is measured by how close the results are to the true value. Some examples of tests of this kind are:

— antibiotic assay of serum;
— measurement of minimal inhibitory concentration (MIC) values of antibiotics in vitro;
— serum antibody titrations.

For tests that give qualitative results, reliability is measured by whether the result is correct. Some examples of tests of this kind are:

— identification of pathogens;
— antibiotic susceptibility testing of isolates by the disc method.

Standard terminology for microorganisms is essential to reliability. Internationally recognized nomenclature should always be used. For example: *Staphylococcus aureus*, NOT "pathogenic staphylococci"; *Streptococcus pyogenes*, NOT "haemolytic streptococci".

Use of uniform, approved methods is essential. For example, disc susceptibility tests should be performed with an internationally recognized technique, such as the modified Kirby–Bauer test (page 109).

Reproducibility

The reproducibility or precision of a microbiological test is reduced by two things:

1. *Lack of homogeneity*. A single sample from a patient may contain more than one organism. Repeat culturing may therefore isolate different organisms.
2. *Lack of stability*. As time passes, the microorganisms in a specimen multiply or die at different rates. Repeat culturing may therefore isolate different organisms. To improve precision, therefore, specimens should be tested as soon as possible after collection.

Efficiency

The efficiency of a microbiological test is its ability to give the correct diagnosis of a pathogen or a pathological condition. This is measured by two criteria:

1. *Diagnostic sensitivity*

$$\text{Sensitivity} = \frac{\text{total number of positive results}}{\text{total number of infected patients}}$$

The greater the sensitivity of a test, the fewer the number of false-negative results.

For example, the sensitivity of MacConkey agar is poor for the isolation of *Salmonella typhi* from stool. This important enteric pathogen is often missed because of overgrowth by nonpathogenic intestinal bacteria.

2. *Diagnostic specificity*

$$\text{Specificity} = \frac{\text{total number of negative results}}{\text{total number of unifected patients}}$$

The greater the specificity of a test, the fewer the number of false-positive results.

For example:

- Ziehl–Neelsen staining of sputum is highly specific for diagnosing tuberculosis, because it gives only a few false-positive results.
- Ziehl–Neelsen staining of urine is much less specific, because it gives many false-positive results (as a result of atypical mycobacteria).
- The Widal test has a very low specificity for the diagnosis of typhoid fever, because cross-agglutinating antibodies remaining from past infections with related salmonella serotypes give false-positive results.

The sensitivity and specificity of a test are interrelated. By lowering the level of discrimination, the sensitivity of a test can be increased at the cost of reducing its specificity, and vice versa. The diagnostic sensitivity and specificity of a test are also related to the prevalence of the given infection in the population under investigation.

Internal quality control

Requirements

An internal quality control programme should be practical, realistic, and economical.

An internal quality control programme should not attempt to evaluate every procedure, reagent, and culture medium on every working day. It should evaluate each procedure, reagent, and culture medium according to a practical schedule, based on the importance of each item to the quality of the test as a whole.

Procedures

Internal quality control begins with proper laboratory operation.

Laboratory operations manual

Each laboratory should have an operations manual that includes the following subjects:

— cleaning of the working space,
— personal hygiene,
— safety precautions,
— designated eating and smoking areas located outside the laboratory,
— handling and disposal of infected material,

— appropriate vaccinations for workers, e.g. hepatitis B,
— care of equipment,
— collection of specimens,
— registration of specimens,
— elimination of unsuitable specimens,
— processing of specimens,
— recording of results,
— reporting of results.

The operations manual should be carefully followed, and regularly revised and updated.

Care of equipment

It is particularly important to take good care of laboratory equipment. Good quality tests cannot be performed if the equipment used is either of poor quality or poorly maintained.

Table 1 is a schedule for the routine care and maintenance of essential equipment. Equipment operating temperatures may be recorded on a form such as the one shown in Fig. 2.

Culture media

Culture media may be prepared in the laboratory from the basic ingredients or from commercially available dehydrated powders, or they may be purchased ready for use. Commercial dehydrated powders are recommended because they are economical to transport and store, and their quality is likely to be higher than media prepared in the laboratory. For best results, careful attention is required to the points itemized below.

Selection of media

An efficient laboratory stocks the smallest possible range of media consistent with the types of test performed. For example, a good agar base can be used as an all-purpose medium for preparing blood agar, chocolate agar, and several selective media.

One highly selective medium (*Salmonella–Shigella* agar or deoxycholate citrate agar) and one less selective medium (MacConkey agar) are necessary for the isolation of pathogenic Enterobacteriaceae from stools.

A special culture medium should be added for the recovery of *Campylobacter* spp.

Ordering and storage of dehydrated media

1. Order quantities that will be used up in 6 months, or at most 1 year.
2. The overall quantity should be packed in containers that will be used up in 1–2 months.
3. On receipt, tighten caps of all containers securely. Dehydrated media absorb water from the atmosphere. In a humid climate, seal the tops of containers of dehydrated media with paraffin wax (fill the space between the lid and container with molten wax, and let it harden).

Table 1. Quality control of equipment

Equipment	Routine care	Monitoring	Technical maintenance and inspection
Anaerobic jar	Clean inside of jar each week Reactivate catalyst after each run (160 °C, 2 h) Replace catalyst every 3 months	Use methylene blue indicator strip with each run Note and record decolorization time of indicator each week	Inspect gasket sealing in the lid weekly
Autoclave	Clean and change water monthly	Check and adjust water level before each run Record time and temperature or pressure for each run Record performance with spore-strips weekly	Every 6 months
Centrifuge	Wipe inner walls with antiseptic solution weekly or after breakage of glass tubes or spillage		Replace brushes annually
Hot-air oven for sterilization of glassware	Clean inside monthly	Record time and temperature for each run	Every 6 months
Incubator	Clean inside walls and shelves monthly	Record temperature at the start of each working day (allowance 35 ± 1 °C)	Every 6 months
Microscope	Wipe lenses with tissue or lens paper after each day's work Clean and lubricate mechanical stage weekly Protect with dust cover when not in use	Check alignment of condenser monthly Place a dish of blue silica with the microscope under the dust cover to prevent fungal growth in humid climates	Annually
Refrigerator	Clean and defrost every 2 months and after power failure	Record temperature every morning (allowance 2–8 °C)	Every 6 months
Water-bath	Wipe inside walls and change water monthly	Check water level daily Record temperature on first day of each week (allowance 55–57 °C)	Every 6 months

4. Write the date of receipt on each container.
5. Store in a dark, cool, well-ventilated place.
6. Rotate the stock so that the older materials are used first.
7. When a container is opened, write the date of opening on it.
8. Discard all dehydrated media that are either caked or darkened.
9. Keep written records of media in stock.

Preparation of media

1. Follow strictly the manufacturer's instructions for preparation.
2. Prepare a quantity that will be used up before the shelf-life expires (see below).

Fig. 2. Record of equipment operating temperature

Temperature _____

Instrument _____ Room _____

Read daily. Check if temperature indication is acceptable. If aberrant, record temperature in space.

Date	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Date
1													1
2													2
3													3
4													4
5													5
6													6
7													7
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9													9
10													10
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Storage of prepared media

1. Protect against sunlight.
2. Protect against heat. Media containing blood, other organic additives, or antibiotics should be stored in the refrigerator.
3. The shelf-life of prepared media, when stored in a cool, dark place, will depend on the type of container used. Typical shelf-lives are:
   — tubes with cotton-wool plugs, 3 weeks;
   — tubes with loose caps, 2 weeks;
   — containers with screw-caps, 3 months;
   — Petri dishes, *if sealed in plastic bags*, 4 weeks.

Quality control of prepared media

1. *pH testing*. The pH of the prepared medium need not be checked routinely when it is correctly prepared from dehydrated powder. If the medium is prepared from basic ingredients, it should be allowed to cool before the pH is tested. Solid media should be tested with a surface electrode or after maceration in distilled water. If the pH differs by more than 0.2 units from the specification, adjust with acid or alkali or prepare a new batch.
2. *Sterility testing*. Carry out routine sterility tests on media to which blood or other components have been added after autoclaving. Take 3–5% of each batch and incubate at 35 °C for 2 days. Refrigerate the rest. If more than two colonies per plate are seen, discard the whole batch.
3. *Performance testing*. The laboratory should keep a set of stock strains for monitoring the performance of media. A suggested list of stock strains is give in Table 2. These strains can be obtained through routine work, or from commercial or official sources. Recommendations for the maintenance and use of stock strains are given on page 14.

A list of performance tests for commonly used media is given in Table 3.

Table 2. Suggested stock strains for quality control[a]

Gram-positive cocci
Enterococcus faecalis (ATCC 29212 or 33186)
Staphylococcus aureus (ATCC 25923)
Staphylococcus epidermidis
Streptococcus agalactiae
Streptococcus mitis
Streptococcus pneumoniae
Streptococcus pyogenes
Gram-negative fastidious organisms
Moraxella catarrhalis
Haemophilus influenzae type b
β-lactamase-negative
β-lactamase-positive
Haemophilus parainfluenzae
Neisseria gonorrhoeae
Neisseria meningitidis
Anaerobes
Bacteroides fragilis
Clostridium perfringens
Enterobacteriaceae
Citrobacter freundii
Enterobacter cloacae
Escherichia coli (ATCC 25922)
Klebsiella pneumoniae
Proteus mirabilis
Salmonella typhimurium
Serratia marcescens
Shigella flexneri
Yersinia enterocolitica
Other Gram-negative rods
Acinetobacter lwoffi
Pseudomonas aeruginosa (ATCC 27853)
Vibrio cholerae (non-01)
Fungi
Candida albicans

[a] The strains most relevant to the needs of the laboratory should be selected.

Table 3. Performance tests on commonly used media

Medium	Incubation	Control organism	Expected result
Bile–aesculin agar	24 h	*Enterococcus faecalis*	Growth and blackening
		α-Haemolytic *Streptococcus*	No growth, with haemolysis
Blood agar	24 h, CO_2	*Streptococcus pyogenes*	Growth and β-haemolysis
		S. pneumoniae	Growth and α-haemolysis
Chocolate agar	24 h, CO_2	*Haemophilus influenzae*	Growth
Decarboxylase (cover with sterile oil)			
— lysine	48 h	*Shigella typhimurium*	Positive
		Shigella flexneri	Negative
— ornithine	48 h	*S. typhimurium*	Positive
		Klebsiella pneumoniae	Negative
Dihydrolase			
— arginine	48 h	*S. typhimurium*	Positive
		Proteus mirabilis	Negative
Gelatinase (rapid tests)	24 h	*Escherichia coli*	Negative
		Serratia marcescens	Positive
Kligler iron agar (see Triple sugar iron agar)			
MacConkey agar with crystal violet	24 h	*E. coli*	Red colonies
		P. mirabilis	Colourless colonies (no swarming)
		E. faecalis	No growth
Malonate broth	24 h	*E. coli*	Negative (green)
		K. pneumoniae	Positive (blue)
Mannitol salt agar	24 h	*Staphylococcus aureus*	Yellow colonies
		Staphylococcus epidermidis	Rose colonies
		E. coli	No growth
Methyl red/Voges–Proskauer	48 h	*E. coli*	Positive/negative
		K. pneumoniae	Negative/positive
Mueller–Hinton agar	24 h	*E. coli* ATCC 25922	Acceptable zone sizes (Table 24, p. 110)
		S. aureus ATCC 25923	
		Pseudomonas aeruginosa ATCC 27853	
Nitrate broth	24 h	*E. coli*	Positive
		Acinetobacter lwoffi	Negative
Oxidation/fermentation dextrose (without oil)	24 h	*P. aeruginosa*	Oxidation at the suface
		A. lwoffi	No change
Peptone water (indole)	24 h	*E. coli*	Positive
		K. pneumoniae	Negative
Phenylalanine deaminase/ ferrichloride	24 h	*E. coli*	Negative
		P. mirabilis	Positive
Salmonella–Shigella agar or deoxycholate citrate agar	24 h	*E. coli*	No growth
		S. typhimurium	Colourless colonies
		Yersina enterocolitica	Colourless colonies
		S. flexneri	Colourless colonies
Selenite broth	24 h	*S. typhimurium*	Growth after subculture
		E. coli	No growth after subculture
Simmons citrate agar (incubate with loose screw-cap)	48 h	*E. coli*	No growth
		K. pneumoniae	Growth, blue colour

Table 3 (continued)

Medium	Incubation	Control organism	Expected result
Thiosulfate citrate bile salts agar	24 h	*Vibrio* spp. (non-agglutinating)	Yellow colonies
Thayer–Martin agar	24 h, CO_2	*Neisseria meningitidis*	Growth
		Neisseria gonorrhoeae	Growth
		Staphylococcus spp.	No growth
		E. coli	No growth
		C. albicans	No growth
Thioglycollate broth	24 h	*Bacteroides fragilis*	Growth
Triple sugar iron agar (depth of butt should be at least 2.5 cm; incubate with loose screw-cap)	24 h	*Citrobacter freundii*	A/A gas[a] + H_2S
		S. typhimurium	K/A gas[a] + H_2S
		S. flexneri	K/A gas[a]
		A. lwoffi	*No change*
Urea medium	24 h	*E. coli*	Negative
		P. mirabilis	Positive (pink)
Voges–Proskauer (see Methyl red/Voges–Proskauer)			

[a]A/A: acid slant; K/A: alkaline slant.

The procedures to be followed when carrying out performance tests on new batches of media are:

1. Prepare a suspension of the stock strain with a barely visible turbidity, equivalent to that of the barium sulfate standard used in the modified Kirby–Bauer method (McFarland 0.5) (see page 109) and use 1 loopful as inoculum.
2. Incubate for the length of time used routinely. Read the plates in the usual way.
3. Keep proper records of results.

Stains and reagents

Recommendations for testing a number of reagents are given in Table 4. Testing should be carried out:
— each time a new batch of working solution is prepared;
— every week (this is critical for the cold Ziehl–Neelsen stain: the classical stain has a shelf-life of several months).

Stains and reagents should be discarded when:

— the manufacturer's expiry date is reached;
— visible signs of deterioration appear (turbidity, precipitate, discoloration).

Diagnostic antigens and antisera

In order to obtain the best results from antigens and antisera:

- Always follow the manufacturer's instructions.
- Store at the recommended temperature. Some serological reagents do not tolerate freezing.

Table 4. Performance tests on commonly used reagents

Reagent or stain	Species suitable for testing		Medium
	Positive	Negative	
Bacitracin disc	*S. pyogenes* (zone)	*E. faecalis*	Blood agar
Catalase	*S. aureus*	*E. faecalis*	Tryptic soy agar
Coagulase plasma	*S. aureus*	*S. epidermidis*	Tryptic soy agar
β-Glucuronidase (PGUA)[a]	*E. coli*	*K. pneumoniae*	Tryptic soy agar
Gram stain	*Staphylococcus* spp	*E. coli*	Mixed in smear
ONPG[b]	*E. coli*	*S. typhimurium*	Triple sugar iron agar or Kligler iron agar
Optochin disc	*S. pneumoniae* (zone)	*Streptococcus mitis*	Blood agar
Oxidase	*Pseudomonas aeruginosa*	*E. coli*	Tryptic soy agar
Tellurite disc	*E. faecalis* (no zone)	*Streptococcus agalactiae* (zone)	Blood agar
V-factor (disc or strips)	*Haemophilus parainfluenzae*	*Haemophilus influenzae*	Tryptic soy agar
XV-factor (disc or strips)	*H. influenzae*		Tryptic soy agar
Ziehl–Neelsen stain	*Mycobacterium tuberculosis*	Mixed non-acid-fast flora	Sputum smear[c]

[a]4-Nitrophenyl-β-D-glucopyranosiduronic acid. (PGUA)
[b]*o*-Nitrophenyl-β-D-galactopyranoside.
[c]Prepare a number of smears from known positive and negative patients. Fix by heat, wrap individually in paper, and store in the refrigerator.

- Avoid repeated freezing and thawing. Before freezing, divide antiserum into aliquot portions sufficient for a few tests.
- Discard when the manufacturer's expiry date is reached.
- To test agglutinating antisera, always use fresh pure cultures of known reactivity.
- Always include a serum control of known reactivity in each batch of tests. The serum may be from a patient, or from a commercial source.
- If possible, the potency of the control serum should be expressed in International Units per millilitre.
- Paired sera from the same patient, taken during the acute and convalescent phases of the disease, should be tested with the same batch of reagents.
- For the serological diagnosis of syphilis, only nationally or internationally recognized procedures should be used.
- Each batch of serological tests should include:
  — a negative serum (specificity control);
  — a weakly reactive serum (sensitivity control);
  — a strongly reactive serum (titration control), which should read within one dilution of its titre when last tested.
- Always record all control serum titres.

Antibiotic susceptibility tests

The routine use of the modified Kirby–Bauer method is recommended (page 109). To avoid errors, the following guidelines should be used:

- Discs should be of correct diameter (6.35 mm).
- Discs should be of correct potency (Table 24, page 110).
- The stock supply should be stored frozen (–20 °C).

- The working supply should be kept no longer than 1 month in a refrigerator (2–8 °C).
- Only Mueller–Hinton agar of performance-tested quality should be used.
- Correct pH (7.2–7.4) of the finished medium is essential for some antibiotics.
- The inoculum should be standardized against the prescribed turbidity standard (page 111).
- Zone sizes should be measured exactly.
- Zone sizes should be interpreted by referring to a table of critical diameters. Zone diameters for each organism should fall within the limits given in Table 24 (page 110).
- The three standard control strains are:[1]
  — *Staphylococcus aureus* (ATCC 25923; NCTC 6571);
  — *Escherichia coli* (ATCC 25922; NCTC 10418);
  — *Pseudomonas aeruginosa* (ATCC 27853; NCTC 10622).
- Tests should be carried out with the three standard strains:
  — when a new batch of discs is put into use;
  — when a new batch of medium is put into use;
  — once a week, in parallel with the routine antibiograms.
- Use the quality control chart shown in Fig. 16 (page 121) for recording and evaluating performance tests.

Maintenance and use of stock cultures

Selection and origin

Select the strains so that the maximum number of morphological, metabolic, and serological characteristics can be tested with the minimum number of cultures; a suggested list is given in Table 2. These strains can be obtained from a combination of the following sources:

— properly documented isolates from clinical specimens;
— official culture collections;
— commercial producers;
— external quality assessment surveys;
— reference laboratories.

Preservation

Long-term preservation

Long-term preservation methods permit intervals of months or even years between subcultures. The best methods are lyophilization (freeze-drying), or storage at –70 °C or below, in an electric freezer or in liquid nitrogen. Alternative methods are described below.

Glycerol at –20 °C

1. Grow a pure culture on an appropriate solid medium.
2. When the culture is fully developed, scrape it off with a loop.
3. Suspend small clumps of the culture in sterile neutral glycerol.

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[1] These strains can be obtained from: American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110, USA; or National Collection of Type Cultures (NCTC), PHLS Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT, England.

4. Distribute in quantities of 1–2 ml in screw-capped tubes or vials.
5. Store at –20 °C. Avoid repeated freezing and thawing. Transfer after 12–18 months.

Mineral oil at room temperature[1]

1. Prepare tubes of heart infusion agar with a short slant. For fastidious organisms, add fresh native or heated blood.
2. Sterilize mineral oil (liquid petrolatum) in hot air (170 °C for 1 hour).
3. Grow a pure culture on the agar slant.
4. When good growth is seen, add sterile mineral oil to about 1 cm above the tip of the slant.
5. Subculture when needed by scraping growth from under the oil.
6. Store at room temperature. Transfer after 6–12 months.

Stab cultures at room temperature (use for non-fastidious organisms only, such as staphylococci and Enterobacteriaceae)

1. Prepare tubes with a deep butt of carbohydrate-free agar. Tryptic soy agar (soybean casein digest agar) is recommended.
2. Stab the organism into the agar.
3. Incubate overnight at 35 °C.
4. Close tube with screw-cap or cork. Dip cap or cork into molten paraffin wax to seal.
5. Store at room temperature. Transfer after 1 year.

Stab cultures in cystine trypticase agar (*CTA*) (for *Neisseria* and streptococci)

1. Prepare tubes of CTA basal medium.
2. Stab the organism into the medium.
3. Incubate overnight at 35 °C.
4. Close tube with screw-cap or cork. Dip cap or cork into molten paraffin wax to seal.
5. For *Neisseria*, store at 35 °C, and transfer every 2 weeks. For streptococci, store at room temperature, and transfer every month.

Cooked-meat medium for anaerobes

1. Inoculate tubes.
2. Incubate overnight at 35 °C.
3. Close tube with screw-cap or cork.
4. Store at room temperature. Transfer every 2 months.

Short-term preservation

Working cultures for daily routine tests can be prepared in the following ways.

Rapid-growing organisms

1. Inoculate on tryptic soy agar slants in screw-capped tubes.
2. Incubate overnight at 35 °C.
3. Store in a refrigerator. Transfer every 2 weeks.

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[1] Morton HE, Pulaski EJ. The preservation of bacterial cultures. *Journal of Bacteriology*, 1938, **38**:163–183.

Streptococci

1. Inoculate on blood agar slants in screw-capped tubes.
2. Incubate overnight at 35 °C.
3. Store in a refrigerator. Transfer every 2 weeks.

Meningococci and Haemophilus

1. Inoculate on chocolate agar slants or plates.
2. Incubate overnight at 35 °C.
3. Store at room temperature. Transfer twice a week.

Gonococci

1. Inoculate on chocolate agar.
2. Incubate and store at 35 °C. Transfer every 2 days.
3. Replace the quality control strain by each new clinical isolate.

Use of reference laboratories

The following categories of specimen should be submitted to a regional or central reference laboratory:

— specimens for infrequently requested or highly specialized tests (e.g. virology, serodiagnosis of parasitic infections);
— occasional duplicate specimens, as a check on the submitting laboratory's own results;
— specimens needing further confirmation, specification, grouping, or typing of pathogens of great public health importance (e.g. *Salmonella*, *Shigella*, *Vibrio cholerae*, *Brucella*, meningococci, and pneumococci).

Reference laboratories should be able to supply reference cultures for quality control and training needs, and standard sera and reagents for comparison with those in use in the referring laboratory.

If no external quality assessment programme exists, the reference laboratory should be asked to supply blind, coded specimens and cultures so that the referring laboratory may test its own proficiency in isolation and identification.

External quality assessment

This section gives information on what is involved in participation in an external quality assessment scheme (sometimes known as a "proficiency testing scheme").

Purposes

The purposes of a quality assessment programme are:

— to provide assurance to both physicians and the general public that laboratory diagnosis is of good quality;
— to assess and compare the reliability of laboratory performance on a national scale;
— to identify common errors;
— to encourage the use of uniform procedures;

— to encourage the use of standard reagents;
— to take administrative measures (which may include revocation of the operating licence) against substandard laboratories;
— to stimulate the implementation of internal quality control programmes.

Organization

A quality assessment programme consists of a number of surveys in which coded specimens are distributed by mail to participating laboratories. These specimens should be incorporated into the laboratory routine, and handled and tested in exactly the same way as routine clinical specimens.

The surveys should be conducted in accordance with the following recommendations:

— surveys should be carried out at least 4 times a year;
— a minimum of 3 specimens should be included in each survey;
— the reporting period should be short, for example 2 weeks following receipt of the specimens;
— instructions and report forms should be included with each survey and the report sheet should be in duplicate, with a clearly stated deadline.

Cultures

Cultures should be included for identification and for susceptibility testing against a limited range of antibiotics; they may be pure cultures or mixtures of two or more cultures.

Cultures should represent at least the first 3 of the following 6 categories:

1. Bacterial species that are of great public health potential, but which are not often seen in routine practice, for example *Corynebacterium diphtheriae*, *Salmonella paratyphi* A.
   NOTE: *Brucella* and *Salmonella typhi* should **not** be used for quality assessment schemes, since they may give rise to serious accidental infections.
2. Abnormal biotypes that are often misidentified, for example H_2S-positive *Escherichia coli*, lactose-negative *E. coli*, urease-negative *Proteus*.
3. Newly recognized or opportunistic pathogens, for example *Yersinia enterocolitica*, *Vibrio parahaemolyticus*, *Burkholderia, Pseudomonas cepacia*.
4. A mixture of *Shigella*, *Citrobacter*, *E. coli*, and *Klebsiella* may be used to test the skill of a laboratory in isolating pathogenic microorganisms from a number of commensal organisms.
5. A mixture of nonpathogenic organisms may be used to test for ability to recognize negative specimens.
6. Bacteria with special resistance patterns, for example meticillin-resistant *S. aureus* (MRSA).

Sera

Serological tests for the following infections should be part of an external quality assessment programme in bacteriology:

— syphilis
— rubella
— brucellosis

— streptococcal infections
— typhoid fever.

Rating and reporting of results

As soon as all reports of results are received from participating, the correct answers should be sent to the laboratories. Within one month after that, a final report should be sent to the laboratories with an analysis of the results. A performance score is given to each laboratory. Each laboratory should have a code number known only to itself. Thus it can recognize its own performance in relation to others, but the other laboratories remain anonymous.

Part I

Bacteriological investigations

Blood

Introduction

Blood is cultured to detect and identify bacteria or other cultivable microorganisms (yeasts, filamentous fungi). The presence of such organisms in the blood is called bacteraemia or fungaemia, and is usually pathological. In healthy subjects, the blood is sterile. However, there are a few exceptions: transient bacteraemia often occurs shortly after a tooth extraction or other dental or surgical manipulation of contaminated mucous membranes, bronchoscopy, or urethral catheterization. This type of transient bacteraemia is generally due to commensal bacteria and usually resolves spontaneously through phagocytosis of the bacteria in the liver and spleen.

Septicaemia is a clinical term used to describe bacteraemia with clinical manifestations of a severe infection, including chills, fever, malaise, toxicity, and hypotension, the extreme form being shock. Shock can be caused by toxins produced by Gram-negative rods or Gram-positive cocci.

When and where bacteraemia may occur

Bacteraemia is a feature of some infectious diseases, e.g. brucellosis, leptospirosis and typhoid fever. Persistent bacteraemia is a feature of endovascular infections, e.g. endocarditis, infected aneurysm and thrombophlebitis.

Transient bacteraemia often accompanies localized infections such as arthritis, bed sores, cholecystitis, enterocolitis, meningitis, osteomyelitis, peritonitis, pneumonia, pyelonephritis, and traumatic or surgical wound infections. It can arise from various surgical manipulations, but usually resolves spontaneously in healthy subjects.

Bacteraemia and fungaemia may result from the iatrogenic introduction of microorganisms by the intravenous route: through contaminated intravenous fluids, catheters, or needle-puncture sites. Both types of infection may develop in users of intravenous drugs and in immunosuppressed subjects, including those with human immunodeficiency virus/the acquired immunodeficiency syndrome (HIV/AIDS). They are often caused by "opportunistic" microorganisms and may have serious consequences. Table 5 shows the most common causes of bacteraemia or fungaemia.

Blood collection

Timing of blood collection

Whenever possible, blood should be taken before antibiotics are administered. The best time is when the patient is expected to have chills or a temperature spike. It is recommended that two or preferably three blood cultures be obtained, separated by intervals of approximately 1 hour (or less if treatment cannot be delayed). More than three blood cultures are rarely indicated. The advantages of repeated cultures are as follows:

— the chance of missing a transient bacteraemia is reduced;
— the pathogenic role of "saprophytic" isolates (e.g. *Staphylococcus epidermidis*) is confirmed if they are recovered from multiple venepunctures.

Table 5. Common causes of bacteraemia or fungaemia

Gram-negative organisms	Gram-positive organisms
Escherichia coli	*Staphylococcus aureus*
Klebsiella spp.	*S. epidermidis*
Enterobacter spp.	α-Haemolytic (viridans) streptococci
Proteus spp.	*Streptococcus pneumoniae*
Salmonella typhi	*E. faecalis* (group D)
Salmonella spp. other than *S. typhi*	*S. pyogenes* (group A)
Pseudomonas aeruginosa	*S. agalactiae* (group B)
Neisseria meningitidis	*Listeria monocytogenes*
Haemophilus influenzae	*Clostridium perfringens*
Bacteroides fragilis (anaerobe)	*Peptostreptococcus* spp. (anaerobes)
Brucella spp.	*Candida albicans* and other yeast-like fungi (e.g. *Cryptococcus neoformans*)
Burkholderia (Pseudomonas) pseudomallei (in certain areas)	

It is important that blood specimens for culture are collected before initiating empirical antimicrobial therapy. If necessary, the choice of antimicrobial can be adjusted when the results of susceptibility tests become available.

Quantity of blood

Because the number of bacteria per millilitre of blood is usually low, it is important to take a reasonable quantity of blood: 10 ml per venepuncture for adults; 2–5 ml may suffice for children, who usually have higher levels of bacteraemia; for infants and neonates, 1–2 ml is often the most that can be obtained. Two tubes should be used for each venepuncture: the first a vented tube for optimal recovery of strictly aerobic microorganisms, the second a non-vented tube for anaerobic culture.

Skin disinfection

The skin at the venepuncture site must be meticulously prepared using a bactericidal disinfectant: 2% tincture of iodine, 10% polyvidone iodine, 70% alcohol, or 0.5% chlorhexidine in 70% alcohol. The disinfectant should be allowed to evaporate on the skin surface before blood is withdrawn. If tincture of iodine is used, it should be wiped off with 70% alcohol to avoid possible skin irritation.

Even after careful skin preparation, some bacteria persist in the deeper skin layers and may gain access to the blood, e.g. *S. epidermidis*, *Propionibacterium acnes*, and even spores of *Clostridium*. Pseudobacteraemia (false-positive blood culture) may result from the use of contaminated antiseptic solutions, syringes, or needles. The repeated isolation of an unusual organism (e.g. *Burkholderia (Pseudomonas) cepacia*, *Pantoea (Enterobacter) agglomerans*, or *Serratia* spp.) in the same hospital must raise suspicion of a nosocomial infection and promote an investigation. Another source of contamination is contact of the needle with non-sterile vials (or solutions), if the same syringe is first used to provide blood for chemical analysis or measurement of the erythrocyte sedimentation rate.

Anticoagulant

The use of sodium polyanethol sulfonate (SPS) as an anticoagulant is recommended because it also inhibits the antibacterial effect of serum and phagocytes. If the blood is immediately added to a sufficient volume (50 ml) of broth and thoroughly mixed to prevent clotting, no anticoagulant is needed. It is recommended that blood-culture bottles be available at all hospitals and major health centres. If blood-culture bottles are not available, blood may be transported to the laboratory in a tube containing a sterile anticoagulant solution (citrate, heparin, or SPS). Upon receipt in the laboratory, such blood samples must be transferred immediately to blood-culture bottles using a strict aseptic technique. Where blood is taken without anticoagulant, the clot can be aseptically transferred to broth in the laboratory and the serum used for certain serological tests (e.g. Widal).

Blood-culture media

Choice of broth medium

The blood-culture broth and tryptic soy broth (TSB) should be able to support growth of all clinically significant bacteria.

Quantity of broth

Ideally, the blood should be mixed with 10 times its volume of broth (5 ml of blood in 50 ml of broth) to dilute any antibiotic present and to reduce the bactericidal effect of human serum.

Blood-culture bottles

Blood-culture bottles (125 ml) with a pre-perforated screw-cap and a rubber diaphragm must be used. Fill the bottle with 50 ml of medium and then loosen the screw-cap half a turn. Cover the cap with a square piece of aluminium foil, and autoclave the bottle for 20 minutes at 120 °C. Immediately after autoclaving, while the bottle and the medium are still hot, securely tighten the cap without removing the aluminium foil (otherwise the cap will not be sterile). As the medium cools, a partial vacuum will be created in the bottle, which will facilitate injection of a blood specimen through the diaphragm.

The top of the cap must be carefully disinfected just before the bottle is inoculated.

Prior to distribution and before use, all blood-culture bottles should be carefully examined for clarity. Any medium showing turbidity should not be used.

If strictly aerobic bacteria (*Pseudomonas*, *Neisseria*) or yeasts are suspected, the bottle should be vented as soon as it is received in the laboratory, by inserting a sterile cotton-wool-plugged needle through the previously disinfected diaphragm. The needle can be removed once the pressure in the bottle reaches atmospheric pressure. Commercial blood-culture bottles often also contain carbon dioxide, which has a stimulating effect on growth.

In countries where brucellosis is prevalent, the use of a diphasic blood-culture bottle, with a broth phase and a solid-slant phase on one of the flat surfaces of the bottle (Castaneda bottle), is recommended for the cultivation of *Brucella* spp. The presence of carbon dioxide is needed for the isolation of most strains of *B. abortus*.

Processing of blood cultures

Incubation time

Blood-culture bottles should be incubated at 35–37 °C and routinely inspected twice a day (at least for the first 3 days) for signs of microbial growth. A sterile culture usually shows a layer of sedimented red blood covered by a pale yellow transparent broth. Growth is evidenced by:

— a floccular deposit on top of the blood layer
— uniform or subsurface turbidity
— haemolysis
— coagulation of the broth
— a surface pellicle
— production of gas
— white grains on the surface or deep in the blood layer.

Whenever visible growth appears, the bottle should be opened aseptically, a small amount of broth removed with a sterile loop or Pasteur pipette, and a Gram-stained smear examined for the presence of microorganisms.

Subcultures are performed by streaking a loopful on appropriate media:

— for Gram-negative rods: MacConkey agar, Kligler iron agar, motility-indole–urease (MIU) medium, Simmons citrate agar;
— for small Gram-negative rods: blood agar;
— for staphylococci: blood agar, mannitol salt agar;
— for streptococci: blood agar with optochin, bacitracin, and tellurite discs, sheep blood agar for the CAMP test, and bile–aesculin agar.

For routine examinations, it is not necessary to incubate blood cultures beyond 7 days. In some cases, incubation may be prolonged for an additional 7 days, e.g. if *Brucella* or other fastidious organisms are suspected, in cases of endocarditis, or if the patient has received antimicrobials.

Blind subcultures and final processing

Some microorganisms may grow without producing turbidity or visible alteration of the broth. Other organisms, e.g. pneumococci, tend to undergo autolysis and die very rapidly. For this reason some laboratories perform routine subcultures on chocolate agar after 18–24 hours of incubation. A blind subculture may be made at the end of 7 days of incubation, by transferring several drops of the well-mixed blood culture (using a sterile Pasteur pipette) into a tube of thioglycollate broth, which in turn is incubated and observed for 3 days.

Antibiogram

When staphylococci or Gram-negative rods are suspected, precious time can be saved by performing a direct, non-standardized antibiogram using the positive broth as an inoculum. A sterile swab is dipped into the turbid broth, excess fluid is expressed, and the swab is used to inoculate Mueller–Hinton medium as in the standard method (see page 110). A provisional reading can often be made after 6–8 hours of incubation. In 95% of cases the results obtained with this method are in agreement with the standardized test.

Contaminants

Contamination of blood cultures can be avoided by meticulous skin preparation and by adherence to strict aseptic procedures for inoculation and subinoculation. However, even in ideal conditions, 3–5% of blood cultures grow "contaminants" originating from the skin (*S. epidermidis*, *P. acnes*, *Clostridium* spp., diphtheroids) or from the environment (*Acinetobacter* spp., *Bacillus* spp.). Such organisms, however, may occasionally behave as pathogens and even cause endocarditis. A true infection should be suspected in the following situations:

— if the same organism grows in two bottles of the same blood specimen;
— if the same organism grows in cultures from more than one specimen;
— if growth is rapid (within 48 hours);
— if different isolates of one species show the same biotypes and antimicrobial-susceptibility profiles.

All culture results should be reported to the clinician, including the presumed contaminants. However, for the latter no antibiogram need be performed and appropriate mention should be made on the report slip, e.g. *Propionibacterium acnes* (skin commensal), *Staphylococcus epidermidis* (probable contaminant). It is to the advantage of all concerned to establish good communication between physicians and laboratory personnel.

The identification of two or more agents may indicate polymicrobial bacteraemia, which can occur in debilitated patients, but may also be due to contamination. "Anaerobic" bacteraemia is often caused by multiple pathogens; for example, one or more anaerobes may be associated with one or more aerobes in severe fulminating bacteraemia associated with severe trauma or surgery involving the large intestine.

Cerebrospinal fluid

Introduction

The examination of cerebrospinal fluid (CSF) is an essential step in the diagnosis of bacterial and fungal meningitis and CSF must always be considered as a priority specimen that requires prompt attention by the laboratory staff.

Normal CSF is sterile and clear, and usually contains three leukocytes or fewer per mm^3 and no erythrocytes. The chemical and cytological composition of CSF is modified by meningeal or cerebral inflammation, i.e. meningitis or encephalitis. Only the microbiological examination of CSF will be discussed here, although the CSF leukocyte count is also of paramount importance.

The most common causal agents of meningitis are listed in Table 6 according to the age of the patient, but it should be kept in mind that some overlap exists.

Collection and transportation of specimens

Approximately 5–10 ml of CSF should be collected in two sterile tubes by lumbar or ventricular puncture performed by a physician. In view of the danger of iatrogenic bacterial meningitis, thorough disinfection of the skin is mandatory. Part of the CSF specimen will be used for cytological and chemical examination, and the remainder for the microbiological examination. The specimen should be delivered to the laboratory at once, and processed immediately, since cells disintegrate rapidly. Any delay may produce a cell count that does not reflect the clinical situation of the patient.

Table 6. Common causes of bacterial and fungal meningitis

In neonates (from birth to 2 months)
- *Escherichia coli*
- *Listeria monocytogenes*
- Other Enterobacteriaceae: *Salmonella* spp., *Citrobacter* spp.
- *Streptococcus agalactiae* (group B)

In all other age groups
- *Haemophilus influenzae* (capsular type b)[a]
- *Neisseria meningitidis*
- *Streptococcus pneumoniae*
- *Mycobacterium tuberculosis*
- *Listeria monocytogenes*[b]
- *Cryptococcus neoformans*[b]
- Staphylococci[c]

[a]Uncommon after the age of 5 years.
[b]In immunocompromised patients (including those with acquired immunodeficiency syndrome (AIDS)).
[c]Associated with neurosurgery and postoperative drains.

Macroscopic inspection

The appearance of the CSF should be noted and recorded as: clear, hazy, turbid, purulent, yellow (due to haemolysis or icterus), or blood-tinged, with fibrin web or pellicle.

Microscopic examination

Preparation of specimen

If, on gross examination, the CSF is purulent (very cloudy), it can be examined immediately without centrifugation. In all other cases, the CSF should be centrifuged in a sterile tube (preferably a 15-ml conical tube with screw-cap) at 10000*g* for 5–10 minutes. Remove the supernatant using a sterile Pasteur pipette fitted with a rubber bulb, and transfer it to another tube for chemical and/or serological tests. Use the sediment for further microbiological tests.

Direct microscopy

Examine one drop of the sediment microscopically (×400), between a slide and coverslip, for:

— leukocytes (polymorphonuclear neutrophils or lymphocytes)
— erythrocytes
— bacteria
— yeasts.

If the yeast-like fungus *Cryptococcus neoformans* is suspected, mix a loopful of the sediment with a loopful of India ink on a slide, place a coverslip on top, and examine microscopically for the typical, encapsulated, spherical, budding yeast forms.

In areas where African trypanosomiasis occurs, it will also be necessary to search carefully for actively motile, flagellated trypanosomes.

A rare and generally fatal type of meningitis is caused by free-living amoebae found in water (*Naegleria fowleri*) which enter through the nose and penetrate the central nervous system. They may be seen in the direct wet preparation as active motile amoebae about the size of neutrophilic leukocytes.

Gram-stained smears

As the causative agent of bacterial meningitis may often be observed in a Gram-stained smear, this examination is extremely important. Air-dry the smear, fix with gentle heat, and stain it by Gram's method. Examine at ×1000 (oil-immersion) for at least 10 minutes, or until bacteria are found. Table 7 lists important diagnostic findings that are associated with different forms of meningitis.

Table 7. Cerebrospinal fluid findings associated with meningitis

Observation	Type of meningitis			
	Bacterial	Tuberculous	Fungal	Viral ("aseptic")
Elevated leukocyte count	Segmented polymorphonuclear neutrophils	Mononuclear (young neutrophils)	Mononuclear	Mononuclear
Glucose	Very low: 0.28–1.1 mmol/l	Low: 1.1–2.2 mmol/l	Low: 1.1–2.2 mmol/l	Normal: 3.6–3.9 mmol/l
Protein	Elevated	Elevated	Elevated	Slightly elevated in early stage of infection
Stained smear	Bacteria usually seen (Gram)	Rarely positive (acid-fast)	Usually positive (India ink)	Negative

Table 8. Choice of culture media for CSF specimens according to the results of the Gram smear[a]

Observation	Gram-negative rods		Gram-positive cocci		Gram-negative cocci	Gram-positive rods	No organisms seen
	In neonates	In other ages	In neonates	In other ages			
Blood agar[b]	+	+	+	+ with optochin disc	+	+	+
Blood agar with *S. aureus* streak[b]	+	+					+
Chocolate agar		(+)			(+)		(+)
MacConkey agar	+	+	+	+	+	+	+
Tryptic soy broth	+	+	+	+	+	+	+

[a] + = Use; (+) = optional use.

[b] Incubate in an atmosphere rich in CO_2 (candle jar).

Acid-fast stain (Ziehl–Neelsen)

Although its sensitivity is not high, examination of an acid-fast-stained preparation of the sediment or of the fibrin web is indicated when tuberculous meningitis is suspected by the physician. Carefully examine the acid-fast-stained preparation for at least 15 minutes. If the result is negative, the microscopic investigation should be repeated on a fresh specimen on the following day.

Culture

If bacteria have been seen in the Gram-stained smear, the appropriate culture media should be inoculated (Table 8). If no organisms have been seen, or if the interpretation of the Gram smear is unclear, it is desirable to inoculate a full range of media, including blood agar with a streak of *Staphylococcus*

aureus to promote growth of *H. influenzae*. Blood agar and chocolate agar plates should be incubated at 35 °C in an atmosphere enriched with carbon dioxide. All media should be incubated for 3 days, with daily inspections.

When tuberculous meningitis is suspected, at least three tubes of Löwenstein–Jensen medium should be inoculated with a drop of the sediment and incubated for 6 weeks. For the first 2–3 days the tubes should be incubated in a horizontal position with the screw-cap loosened half a turn. Tubes should be inspected for growth at weekly intervals. Smears from any suspicious growth should be prepared, preferably in a bacteriological safety cabinet, air-dried, heat-fixed, and stained by the Ziehl–Neelsen method. The presence of acid-fast rods is consistent with the diagnosis of tuberculosis. All isolates should be forwarded to a central laboratory for confirmation and for susceptibility testing.

When *Cryptococcus neoformans* is suspected, either from the India ink preparation or on clinical grounds, the sediment should be inoculated on two tubes of Sabouraud dextrose agar, and incubated at 35 °C for up to 1 month. *C. neoformans* also grows on the blood agar plate, which should be incubated at 35 °C for 1 week, if indicated.

Preliminary identification

Growth on MacConkey agar is suggestive of Enterobacteriaceae and should be further identified using the methods and media recommended for enteric pathogens.

Colonies of Gram-positive cocci with a narrow zone of β-haemolysis may be *S. agalactiae* (group B streptococci). This should be confirmed with the reverse CAMP test (page 101).

Flat colonies with a concave centre and a slight green zone of α-haemolysis are probably *S. pneumoniae*. For confirmation, a 6-mm optochin disc should be placed on a blood agar plate heavily inoculated with a pure culture of the suspected strain. After overnight incubation, pneumococci will exhibit an inhibition zone of 14 mm or more around the optochin disc. The best results are obtained after incubation on sheep blood agar in a carbon-dioxide-enriched atmosphere. If the reading of this test on the primary blood agar plate is inconclusive, the test should be repeated on a subculture.

Colonies of *H. influenzae* will grow only on chocolate agar, and as satellite colonies in the vicinity of the staphylococcal streak on blood agar. Further identification may be accomplished using *H. influenzae* type b antiserum in the slide agglutination test.

Gram-negative diplococci growing on blood and chocolate agar, and giving a rapidly positive oxidase test, may be considered to be meningococci. Confirmation is accomplished by grouping with appropriate *N. meningitidis* antisera (A, B, C) in the slide agglutination test. A negative agglutination test does not rule out meningococci as there are at least four additional serogroups. If the agglutination test is negative, carbohydrate utilization tests should be performed and the culture sent to a central reference laboratory. A preliminary report should be given to the physician at each stage of identification (Gram-stain, growth, agglutination, etc.), noting that a final report will follow.

Colonies of Gram-positive rods with a narrow zone of β-haemolysis on blood agar may be *Listeria monocytogenes*. The following confirmatory tests are

suggested: positive catalase reaction, motility in broth culture or in MIU, growth and black discoloration on bile–aesculin agar.

Susceptibility testing

For Gram-negative rods and staphylococci, the standardized disc-diffusion method (Kirby–Bauer) should be used.

No susceptibility testing is needed for *Listeria monocytogenes*, *S. agalactiae* or *N. meningitidis* since resistance to ampicillin and benzylpenicillin is extremely rare.

All strains of pneumococci should be tested on blood agar for susceptibility to chloramphenicol and benzylpenicillin. For the latter, the oxacillin (1 µg) disc is recommended (see page 66, "Lower respiratory tract infections").

Strains of *H. influenzae* should be tested for susceptibility to chloramphenicol using chocolate agar or a supplemented blood agar. Most ampicillin-resistant strains produce β-lactamase that can be demonstrated using one of the rapid tests recommended for the screening of potential β-lactamase-producing strains of gonococci (page 79).

Urine

Introduction

Urine is the specimen most frequently submitted for culture. It also presents major problems in terms of proper specimen collection, transport, culture techniques, and interpretation of results. As with any other specimen submitted to the laboratory, the more comprehensive the information provided by the submitting physician the more able the laboratory is to provide the best possible culture data.

The most common sites of urinary tract infection (UTI) are the urinary bladder (cystitis) and the urethra. From these sites the infection may ascend into the ureters (ureteritis) and subsequently involve the kidney (pyelonephritis). Females are more prone to infection of the urinary tract than are males and also present the greater problem in the proper collection of specimens.

In both males and females, UTI may be asymptomatic, acute, or chronic. Asymptomatic infection can be diagnosed by culture. Acute UTI is more frequently seen in females of all ages; these patients are usually treated on an outpatient basis and are rarely admitted to hospital. Chronic UTI in both males and females of all ages is usually associated with an underlying disease (e.g. pyelonephritis, prostatic disease, or congenital anomaly of the genitourinary tract) and these patients are most often hospitalized. Asymptomatic, acute, and chronic UTI are three distinct entities and the laboratory results often require different interpretation.

Asymptomatic pyelonephritis in females may remain undetected for some time, and is often only diagnosed by carefully performed quantitative urine culture. Chronic prostatitis is common and difficult to cure, and is often responsible for recurring UTI. In most UTI, irrespective of type, enteric bacteria are the etiological agents, *Escherichia coli* being isolated far more frequently than any other organism. In about 10% of patients with UTI, two organisms may be present and both may contribute to the disease process. The presence of three or more different organisms in a urine culture is strong presumptive evidence of improper collection or handling of the urine specimen. However, multiple organisms are often seen in UTI in patients with indwelling bladder catheters.

Specimen collection

The importance of the method of collection of urine specimens, their transport to the laboratory, and the initial efforts by the laboratory to screen and culture the urine cannot be overemphasized. It is the responsibility of the laboratory to provide the physician with sterile, wide-mouthed, glass or plastic jars, beakers, or other suitable receptacles. They should have tight-fitting lids or be covered with aluminium foil prior to sterilization by dry heat or autoclaving.

Urine specimens may have to be collected by a surgical procedure, e.g. suprapubic aspiration, cystoscopy, or catheterization. If not, the laboratory must insist on a clean-catch midstream urine specimen, particularly in females and children. Since urine itself is a good culture medium, all specimens should be processed by the laboratory within 2 hours of collection, or be kept refriger-

ated at 4 °C until delivery to the laboratory and processed no longer than 18 hours after collection.

Whenever possible, urine specimens for culture should be collected in the morning. It is advisable to ask the patient the night before to refrain from urinating until the specimen is to be collected.

A female outpatient should:

1. Wash her hands thoroughly with soap and water and dry them with a clean towel.
2. Spread the labia, and cleanse the vulva and labia thoroughly using sterile cotton gauze pads and warm soapy water, wiping from front to rear. Disinfectants should not be used.
3. Rinse the vulva and labia thoroughly with warm water and dry with a sterile gauze pad. During the entire process the patient should keep the labia separated and should not touch the cleansed area with the fingers.
4. Pass a small amount of urine. The patient should collect most of the remaining urine in a sterile container, closing the lid as soon as the urine has been collected. This is a midstream urine specimen.
5. Hand the closed container to the nursing personnel for prompt delivery to the laboratory.

A male outpatient should:

1. Wash his hands thoroughly with soap and water and dry them with a clean towel.
2. Pull back the foreskin (if not circumcised) and wash the glans thoroughly using sterile cotton gauze pads and warm soapy water. Disinfectants should not be used.
3. Rinse the glans thoroughly with warm water and dry with a sterile gauze pad. During the entire procedure the patient should not touch the cleansed area with the fingers.
4. Pull back the foreskin and pass a small amount of urine. Still holding back the foreskin, the patient should pass most of the remaining urine into a sterile container, closing the lid as soon as the urine has been collected. This is a midstream urine specimen.
5. Hand the closed container to the nursing personnel for prompt delivery to the laboratory.

For bedridden patients, the same procedure is followed, except that a nurse must assist the patient or, if necessary, do the entire cleansing procedure before requesting the patient to pass urine.

In both situations every effort must be made to collect a clean-catch urine specimen in a sterile container and to ensure that it is delivered promptly to the laboratory together with information on the patient, the clinical diagnosis, and the requested procedures.

Infants and children

Collection of a clean-catch urine specimen from infants and children who are ill in bed or uncooperative can be a problem. Give the child water or other liquid to drink. Clean the external genitalia. The child can be seated on the lap of the mother, nurse, or ward attendant, who should then encourage the child to urinate and collect as much urine as possible in a sterile container. The container should then be covered and delivered to the laboratory for immediate processing.

Culture and interpretation

All urine specimens brought to the microbiology laboratory should be examined at once, or placed in a refrigerator at 4°C until they can be examined. The examination procedure includes the following steps:

1. Examination of a Gram-stained smear.
2. A screening test for significant bacteriuria.
3. A definitive culture for urine specimens found to be positive in the screening test, and for all specimens obtained by cystoscopy, suprapubic bladder puncture (SBP), or catheterization.
4. Susceptibility tests on clinically significant bacterial isolates.

Preparation and examination of a Gram-stained smear is a necessary part of the laboratory process. Using a sterile Pasteur pipette (one for each sample), place one drop of well-mixed, uncentrifuged urine on a slide. Allow the drop to dry without spreading, heat-fix and stain. Examine under an oil-immersion lens (×600 or more) for the presence or absence of bacteria, polymorphonuclear leukocytes, and squamous epithelial cells.

One or more bacterial cells per oil-immersion field usually implies that there are 10^5 or more bacteria per millilitre in the specimen. The presence of one or more leukocytes per oil-immersion field is a further indication of UTI. Non-infected urine samples will usually show few or no bacteria or leukocytes in the entire preparation. In specimens from females, the presence of many squamous epithelial cells, with or without a mixture of bacteria, is strong presumptive evidence that the specimen is contaminated with vaginal flora and a repeat specimen is necessary, regardless of the number of bacteria per oil-immersion field. If results are required urgently, the report of the Gram-stain findings should be sent to the physician with a note that the culture report is to follow.

Screening method

The absence of leukocytes and bacteria in a Gram-stained smear of a clean-catch urine sample prepared as described above is good evidence that the urine is not infected. A urine specimen that is "negative" on careful examination of the Gram-stained smear does not need to be cultured. An alternative simple and effective screening test is the test strip for leukocyte esterase/nitrate reduction. The strip is dipped into the urine specimen as instructed in the package literature. Any pink colour is a positive reaction indicating the presence of leukocyte esterase and/or bacteria in excess of 10^5 per ml. Urine samples that are positive in the screening test should be cultured as soon as possible to prevent possible overgrowth by non-significant bacteria. If the strip does not develop a pink colour it is interpreted as a negative screening test, is so reported, and no culture is indicated. The test strip may not be sensitive enough to detect bacterial counts of less than 10^5 per ml of urine.

Quantitative culture and presumptive identification

Two techniques are recommended here for quantitative culture and presumptive identification: the calibrated loop technique and the filter-paper dip strip method.

Calibrated loop technique

The recommended procedure uses a calibrated plastic or metal loop to transfer 1 µl of urine to the culture medium (MacConkey agar with crystal violet and non-selective blood agar).

1. Shake the urine gently, then tip it to a slant and with a 1-µl inoculating loop touch the surface so that the urine is sucked up into the loop. Never dip the loop into the urine.
2. Deposit 1 µl of the urine on a blood agar plate and streak half the plate by making a straight line down the centre (1), followed by close passes at right angles through the original (2), and ending with oblique streaks crossing the *two previous passes* (3) (Fig. 3).
3. Inoculate the MacConkey agar in the same manner.
4. Incubate the plates overnight at 35 °C.

Blood agar and MacConkey agar can also be replaced by another non-selective medium (e.g. CLED[1] agar, purple lactose agar).

Filter-paper dip-strip method

The filter-paper dip-strip method of Leigh & Williams[2] is based on the absorption and subsequent transfer of a fixed amount of urine to a suitable plating agar medium.

The strips can be locally prepared using a specific type of blotting-paper and should measure 7.5 cm long by 0.6 cm wide (see Fig. 4). They are marked at 1.2 cm from one end with a pencil. The filter-paper dip strip technique should be compared with the calibrated loop technique before adopting the strips for

Fig. 3. Inoculation of bacteria on culture plates

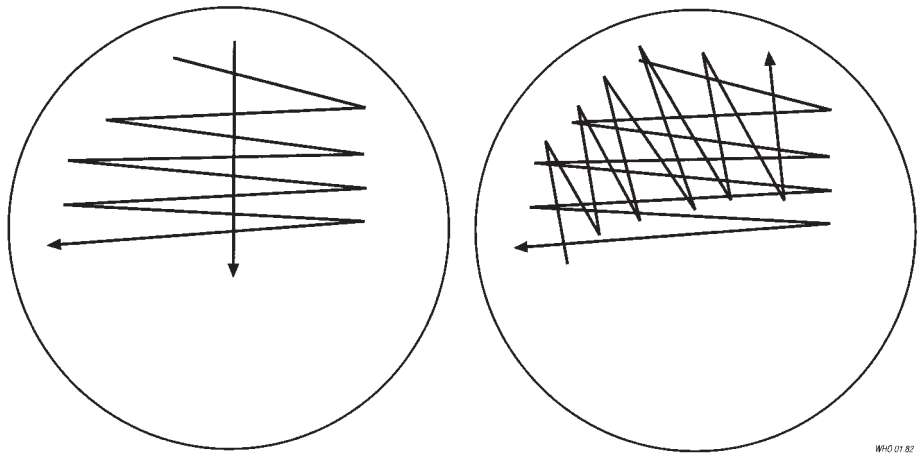
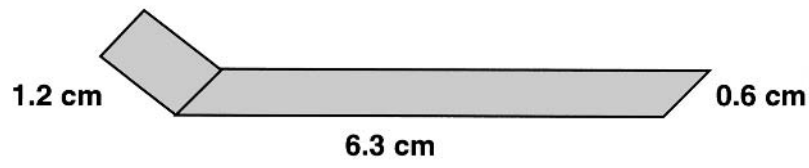


Fig. 4. Diagram of a dip strip



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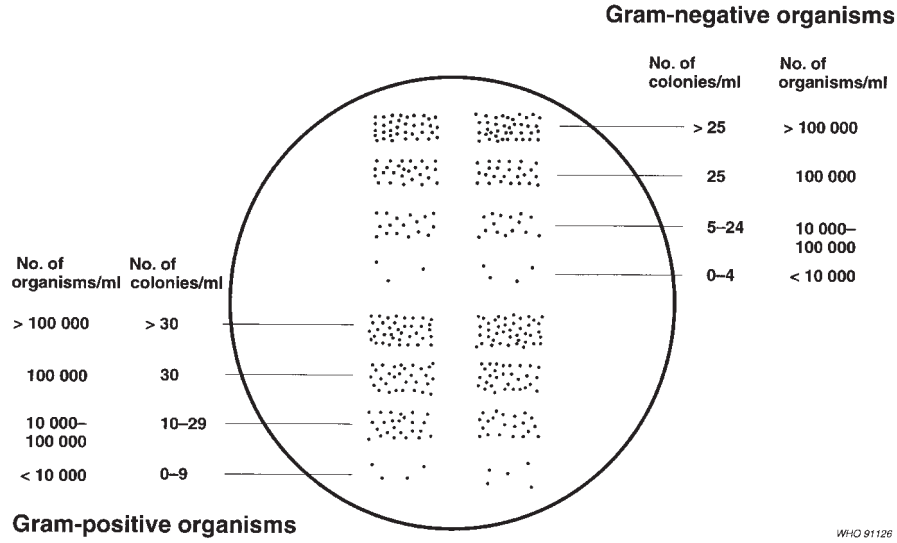
[1] CLED: Cystine-Lactose-Electrolyte Deficient.
[2] Leigh DA & Williams JD. Method for the detection of significant bacteriuria in large groups of patients. *Journal of Clinical Pathology*, 1964, **17**: 498–503.

Fig. 5. Dip-strip impressions on an agar plate, showing conversion from number of colonies to number of bacteria per ml



routine investigations. The strips are made in quantity, placed in a suitable container and autoclaved. Sterile strips are commercially available. A sterile strip is removed from the container for each urine sample to be tested. The marked end is dipped as far as the mark into the thoroughly mixed urine sample. The strip is withdrawn immediately and the excess urine is allowed to be absorbed.

The area below the mark, which will bend over like the foot of an "L", should then be placed in contact with a plate of brolacin agar[1] or purple lactose agar for 2–3 seconds. Several strips can be cultured on one plate by dividing the undersurface of the plate into up to 16 rectangles (Fig. 5). Be sure to identify each rectangular area with the number or name of the patient. Remove a second strip from the container and repeat the procedure exactly, making a second imprint identical to the first. Once the plate is completely inoculated with duplicate impressions, it should be incubated at 35–37 °C, and the colonies resulting from each dip-strip imprint counted. With the help of Fig. 5 it is possible to convert the average number of colonies for each pair of dip-strips to the number of bacteria per ml of urine.

Immediately following the above procedure, inoculate half a plate of MacConkey agar (with crystal violet) with the urine specimen using a sterile loop. The inclusion of a blood agar facilitates the rapid identification of Gram-positive cocci. Plates should be incubated at 35–37 °C overnight, and examined on the following day for growth. Identification procedures may then be initiated using well-separated colonies of similar appearance. If required, the inoculum for performing the disc-diffusion susceptibility test (page 97) can be prepared from either of these plates. In this way, the results of both identification and susceptibility tests will be available on the next day.

Interpretation of quantitative urine culture results

For many years, only the presence of at least 10^5 colony-forming units (CFU) per ml in a clean-catch midstream urine specimen was considered clinically

[1] Bromothymol-blue lactose cystine agar.

relevant for a diagnosis of urinary tract infection. This assumption has been challenged; some experts feel that 10^4 CFU or even fewer may indicate infection. Others believe that the presence of polymorphonuclear leukocytes plays an important role in the pathology and clinical manifestations of UTI. It is not possible to define precisely the minimum number of bacteria per millilitre of urine that is definitely associated with UTI. General recommendations for reporting are given below.

Category 1: fewer than 10^4 CFU per ml. Report as *probable absence* of UTI. (Exceptions: if fewer than 10^4 CFU per ml are present in urine taken directly from the bladder by suprapubic puncture or cystoscopy, in; symptomatic women, or in the presence of leukocytura, report the identification and the result of the susceptibility test.)

Category 2: 10^4–10^5 CFU per ml. If the patient is asymptomatic, request a second urine specimen and repeat the count. If the patient has symptoms of UTI, proceed with both identification and susceptibility tests if one or two different colony types of bacteria are present. Bacterial counts in this range strongly suggest UTI in symptomatic patients, or in the presence of leukocyturia. If the count, the quality of the urine specimen, or the significance of the patient's symptoms is in doubt, a second urine specimen should be obtained for retesting. Report the number of CFU.

Category 3: More than 10^5 CFU per ml. Report the count to the physician and proceed with identification and susceptibility tests if one or two different colony types of bacteria are present. These bacterial counts are strongly suggestive of UTI in all patients, including asymptomatic females.

If more than two species of bacteria are present in urine samples in categories 2 and 3, report as "Probably contaminated; please submit a fresh, clean-catch specimen".

Identification

Identification should be performed as rapidly as possible. Since the vast majority of urinary tract infections are caused by *E. coli*, a rapid test should be used to identify red colonies from MacConkey agar.

β-Glucuronidase test for rapid identification of *E. coli*[1]

This test determines the ability of an organism to produce the enzyme β-glucuronidase. The enzyme hydrolyses the 4-nitrophenyl-β-D-glucopyranosiduronic acid (PGUA) reagent to glucuronic acid and *p*-nitrophenol. The development of a yellow colour indicates a positive reaction.

[1] Kilian M, Borrow P. Rapid diagnosis of Enterobacteriaceae. 1. Detection of bacterial glycosidases. *Acta Pathologica et Microbiologica Scandinavica*, Section B, 1976, **84**:245–251.

Procedure:

1. Prepare a dense milky suspension of the organism to be tested in a small tube containing 0.25 ml of saline. The suspension should be prepared from colonies growing on MacConkey agar.
2. Dissolve 300 mg of 4-nitrophenyl-β-D-glucopyranosiduronic acid (PGUA) and 100 mg of yeast extract (Oxoid L21)[1] in 20 ml of phosphate buffer (Tris buffer, pH 8.5). Adjust the pH to 8.5 ± 0.1. Pour 0.25 ml of the medium into each of the required number of sterile tubes. Close the tubes with stoppers. Label the tubes PGUA and indicate the date.
3. Inoculate one tube of the PGUA medium with a dense suspension of the organism to be tested. Incubate the tube for 4 hours at 35 °C. The development of a yellow colour indicates the presence of β-glucuronidase (positive result); if no colour change occurs the result is negative. The presence of a pigmented yellow colour indicates that the result is unreliable; in such cases other tests must be used.

   Quality control organisms:

Escherichia coli	positive (yellow) result
Shigella flexneri	negative (clear) result

PGUA tablets are commercially available.

Susceptibility tests

Susceptibility tests (page 97) should only be performed on well-isolated colonies of similar appearance that are considered significant according to the guidelines presented above. Susceptibility tests are generally more important on cultures obtained from patients who are hospitalized or have a history of recurring UTI. Cultures from patients a primary UTI may not require a susceptibility test.

[1] Available from Oxoid Ltd, Wade Road, Basingstoke, Hampshire, RG24 8PW, England.

Stool

Introduction

Enteric bacterial infections, causing diarrhoea, dysentery, and enteric fevers, are important health problems throughout the world. Diarrhoeal infections are second only to cardiovascular diseases as a cause of death, and they are the leading cause of childhood death. In developing countries, diarrhoeal; diseases account for 1.5 million death each year among children aged 1–4 years. The risk of children in this age group dying from diarrhoeal disease is 600 times greater in developing countries than in developed countries. In some developing countries, children suffer ten or more episodes of diarrhoea a year.

Children are frequently infected with multiple pathogens and even children without diarrhoea quite often carry potential pathogens in their stools. Observations in studies suggested that active immunization through repeated exposure and prolonged breastfeeding may protect against the diarrhoeagenic effect of these agents. They have also underlined the difficulties in determining the cause of an episode of diarrhoea by culture of a single stool specimen.

With the increasing prevalence of HIV/AIDS and immunosuppressive chemotherapy, diarrhoea in immunocompromised patients has become a growing challenge. Patients with HIV/AIDS either present with diarrhoea or have diarrhoea in a later stage of the disease, and the infection is often life-threatening and difficult to cure. The list of enteric bacterial infections identified in HIV/AIDS patients is long and includes *Campylobacter*, *Salmonella*, *Shigella*, and mycobacteria. Salmonellosis has been estimated to be nearly 20 times as common and 5 times more often bacteraemic in patients with HIV/AIDS than in those without the disease. In Zaire, 84% of patients with diarrhoea of more than a month's duration were found to be seropositive for HIV, and 40% of patients with HIV/AIDS had persistent diarrhoea.

Etiological agents and clinical features

The genus *Salmonella* contains more than 2000 serotypes. Many of these may infect both humans and domestic animals. In humans they cause gastroenteritis, typhoid fever, and bacteraemia with or without metastatic disease. *Salmonella* gastroenteritis usually begins with nausea, vomiting, abdominal colic and diarrhoea 8–48 hours after ingestion of the contaminated food. The symptoms often persist for 3–5 days before resolving without therapy. Antimicrobials will not hasten clinical recovery, and may lengthen the convalescence and asymptomatic carrier state. Antimicrobial susceptibility testing and antimicrobial therapy are not recommended for uncomplicated cases. Antimicrobial treatment is only indicated if the patient appears bacteraemic. Some patients may harbour *Salmonella* spp. in stool or urine for periods of 1 year or longer but remain asymptomatic. Approximately 3% of patients with typhoid fever and 0.2–0.6% of persons with non-typhoid *Salmonella* gastroenteritis will have positive stool cultures for more than 1 year.

Shigella spp. cause a wide spectrum of clinical diseases, which vary from asymptomatic infections to diarrhoea without fever to severe dysentery. Symptoms consist of abdominal cramps, ineffectual and painful straining to pass stool (tenesmus), and frequent, small-volume, bloodstained inflammatory discharge. *Shigella* spp. are the main cause of bacillary dysentery followed by enteroinvasive and enterohaemorrhagic *E. coli*. Many cases present as mild

illnesses that require no specific treatment. However, for severe dysentery or when secondary spread is likely, antimicrobial therapy after susceptibility testing is indicated as resistance towards commonly used antimicrobials is high in many countries. Four groups of *Shigella* organisms, with a total of 39 serotypes and subtypes, are recognized. Group A (*S. dysenteriae*), group B (*S. flexneri*), and group C (*S. boydii*) contain multiple serotypes; there is only one serotype for group D (*S. sonnei*). *S. dysenteriae* and *S. flexneri* are the most commonly isolated *Shigella* species in developing countries, while *S. sonnei* is the most commonly isolated species in developed countries.

At least six different classes of diarrhoea-producing *Escherichia coli* have been identified: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic or verotoxin-producing *E. coli* (EHEC or VTEC), enteroinvasive *E. coli* (EIEC), enteroadhesive *E. coli* (EAEC), and enteroaggregative *E. coli* (EAggEC). Four of them are common causes of diarrhoeal disease in developing countries. However, identification of these strains requires serological assays, toxicity assays in cell culture, pathogenicity studies in animals and gene-probe techniques that are beyond the capacity of intermediate-level clinical laboratories. It may be possible to have a presumptive identification of a VTEC strain, as the most frequent VTEC serotype O157:H7 is characterized by being sorbitol-negative. However, *E. fergusonii* and *E. hermanii* are also sorbitol-negative. A sorbitol-negative *E. coli* strain will need additional identification by serotyping with *E. coli* O157 antiserum. Demonstration of verotoxin production indicates that it is a VTEC strain.

Cholera is a typical example of a toxigenic infection. All the symptoms can be attributed to the intestinal fluid loss caused by an enterotoxin released by *Vibrio cholerae* in the intestine. The stool is voluminous and watery, and contains no inflammatory cells. The main objective of treatment is fluid replacement and antimicrobials have only a secondary role.

V. cholerae spreads very rapidly. It can be divided into several serotypes on the basis of variation in the somatic O antigen, and serotype O1 exists in two biological variants termed "classical" and "El Tor". Until 1992, only *V. cholerae* serogroup O1 (classical or El Tor) was known to produce epidemic cholera, and other serogroups were thought to cause sporadic cholera and extraintestinal infections. However, in 1992 a large outbreak of cholera appeared on the east coast of India and quickly spread to neighbouring countries. This epidemic was caused by a previously unrecognized serogroup of *V. cholerae* O139 Bengal. Isolation of *V. cholerae* O139 has so far been reported from 10 countries in south-east Asia but seems to be on the decline.

V. parahaemolyticus and several other species of *Vibrio* (*V. fluvialis*, *V. hollisae*, *V. mimicus*) and *Aeromonas* (*A. hydrophila*, *A. sobria*, *A. caviae*) cause food poisoning or gastroenteritis in persons who eat raw or undercooked seafood.

Campylobacter jejuni and *C. coli* have emerged as major enteric pathogens that can be isolated as often as *Salmonella* and *Shigella* spp. in most parts of the world. Investigations in Africa and Asia have shown incidence rates of between 19 and 38%, and asymptomatic carrier rates of between 9 and 40%, in children. The intestinal disease varies from a brief, self-limiting enteritis to a fulminant enterocolitis with severe diarrhoea, abdominal colic, fever, and muscle pain. The stools are at first mucoid and liquid and may progress to profuse watery diarrhoea containing blood and pus. Symptoms usually subside within a week. Relapse occurs in about 25% of patients, but is generally milder than the initial episode. The infection is usually self-limiting without antimicrobial therapy, and susceptibility testing is usually not indicated.

Arcobacter butzleri, formerly regarded as a low-temperature-growing *Campylobacter*, has recently been recognized as a cause of diarrhoeal disease in patients, mainly children, in developing countries.

Human infections with *Yersinia enterocolitica* have been reported mainly from northern Europe, Japan and the United States of America. The majority of isolates have been identified from children with sporadic diarrhoea.

Clostridium difficile is the primary cause of enteric disease related to antimicrobial therapy. It produces a broad spectrum of diseases ranging from mild diarrhoea to potentially fatal pseudomembranous colitis. Observation of colonic pseudomembranes by colonoscopy is diagnostic for pseudomembranous colitis, in which case laboratory confirmation is unnecessary. Several commercial tests are available for the laboratory, including culture, latex agglutination for detection of a cell-associated protein, ELISA assay for cytotoxin and/or enterotoxin, and cell culture toxicity assay for cytotoxin. Many hospital patients harbour the organism in the stool in the absence of symptoms, particularly if they are receiving broad-spectrum antimicrobials. Therefore, a culture without demonstration of toxin production has little diagnostic value.

Rotavirus is the only non-bacterial agent to be discussed here. Other viruses are important causes of diarrhoea but rotavirus is common everywhere in the world with similar rates in both developed and developing countries. Most infections occur in children between 6 and 18 months, with a higher prevalence during the cooler months of the year. The diagnosis is made either with an enzyme immunoassay (ELISA) or more simply and practically with a latex agglutination assay. Reagents for both assays are commercially available but are expensive.

Appropriate use of laboratory resources

The laboratory associated with a busy hospital or clinic in a developing country can quickly become overwhelmed with specimens. Cultures are not required for effective management of the majority of cases of diarrhoea and dysentery and patients will require only rehydration and not antimicrobials. A few patients (e.g. those with typhoid fever) will need the results of a culture for appropriate treatment. The problem of how best to utilize scarce resources is a constant concern.

Often, the public health aspects are the most important, and the laboratory must be able to provide data that describe the common enteric pathogens in the district and the antimicrobial susceptibility patterns of these pathogens, and investigate an epidemy. Clinicians must work closely with the laboratory. A procedure that allows the laboratory to develop a valid database is the collection of specimens from a random systematic sample of patients with diarrhoea at the hospital or clinic. By testing only a proportion of these, the number of specimens is reduced and a more complete investigation can be made on each specimen. If the sample is a systematic one (e.g. every twenty-fifth patient), then the results can be used to estimate the infection in the entire population of patients. If a typical clinical syndrome is observed in a particular age group, or during a particular season, the laboratory can focus its sampling on these specific problems.

The laboratory may decide to test for only certain enteric pathogens. *Yersinia enterocolitica* is evidently rare in most tropical areas. If the organism does not occur in the area served by the laboratory, testing for it can be omitted. When

the laboratory provides a report, it should specify which organisms were investigated. If *Salmonella* and *Shigella* species were the only pathogens ruled out, the report should not state "No pathogens found". Rather, it should state "*Salmonella* and *Shigella* species not found".

Collection and transport of stool specimens

Faecal specimens should be collected in the early stages of the diarrhoeal disease, when pathogens are present in the highest number, and preferably before antimicrobial treatment is started, if appropriate. The specimen should be collected in the morning to reach the laboratory before noon, so that it can be processed the same day. Formed stools should be rejected. Ideally, a fresh stool specimen is preferred to a rectal swab, but a rectal swab is acceptable if the collection cannot be made immediately or when transportation of the stool to the laboratory is delayed.

Procedure for collecting stool samples

Provide the patient with two small wooden sticks and a suitable container with a leakproof lid (e.g. a clean glass cup, a plastic or waxed-cardboard box, or a special container with a spoon attached to the lid). The use of penicillin bottles, matchboxes and banana leaves should be discouraged as they expose the laboratory staff to the risk of infection.

Instruct the patient to collect the stool specimen on a piece of toilet tissue or old newspaper and to transfer it to the container, using the two sticks.

The specimen should contain at least 5 g of faeces and, if present, those parts that contain blood, mucus or pus. It should not be contaminated with urine. Once the specimen has been placed in the specimen container, the lid should be sealed.

The patient should be asked to deliver the specimen to the clinic immediately after collection. If it is not possible for the specimen to be delivered to the laboratory within 2 hours of its collection, a small amount of the faecal specimen (together with mucus, blood and epithelial threads, if present) should be collected on two or three swabs and placed in a container with transport medium (Cary–Blair, Stuart or Amies) or 33 mmol/l of glycerol–phosphate buffer. For cholera and other *Vibrio* spp., alkaline peptone water is an excellent transport (and enrichment) medium. Pathogens may survive in such media for up to 1 week, even at room temperature, although refrigeration is preferable.

Procedure for collecting rectal swabs

1. Moisten a cotton-tipped swab with sterile water. Insert the swab through the rectal sphincter, rotate, and withdraw. Examine the swab for faecal staining and repeat the procedure until sufficient staining is evident. The number of swabs to be collected will depend on the number and types of investigation required.
2. Place the swab in an empty sterile tube with a cotton plug or screw-cap, if it is to be processed within 1–2 hours. If the swab must be kept for longer than 2 hours, place it in transport medium.

Visual examination of stool specimens

1. Examine the stool sample visually and record the following:
   — its consistency (formed, unformed (soft) or liquid)
   — its colour (white, yellow, brown or black)
   — the presence of any abnormal components (e.g. mucus or blood).

2. Place a small fleck of the stool specimen or rectal swab together with a small flake of mucus (if present) in a drop of 0.05% methylene blue solution on a clean slide and mix thoroughly.

3. Place a coverslip on the stained suspension, avoiding the formation of air bubbles. Wait 2–3 minutes to. Examine the slide under the microscope using the high-power objective (×100).

4. Record cells that can be clearly identified as mononuclear or polymorphonuclear; ignore degenerated cells.

   Examination of the cellular exudate of diarrhoeal stools may give an indication of the organism involved:

   — clumps of polymorphonuclear leukocytes (>50 cells per high-power field), macrophages and erythrocytes are typical of shigellosis;
   — smaller numbers of polymorphonuclear leukocytes (<20 cells per high-power field) are found in salmonellosis, and invasive *E. coli*. In amoebic dysentery the cells are mostly degenerated (ghost cells). Leukocytes and erythrocytes are also found in about half the cases of diarrhoea due to *Campylobacter* spp.;
   — few leukocytes (2–5 cells per high-power field) are present in cases of cholera, enterotoxigenic and enteropathogenic *E. coli*, and viral diarrhoea.

Enrichment and inoculation of stool specimens

Enrichment is commonly used for the isolation of *Salmonella* spp. and *Vibrio cholerae* from faecal specimens. Selenite F or tetrathionate broths are recommended for the enrichment of *Salmonella* spp., and alkaline peptone water (APW) for the enrichment of *V. cholerae*. Enrichment is not required for *Shigella* spp., *Campylobacter* spp., *Yersinia enterocolitica* and *Clostridium difficile*.

Procedure for inoculation of enrichment media

1. Prepare a faecal suspension by suspending approximately 1 g of the stool sample in a tube containing 1 ml of sterile saline. If the stool sample is liquid, saline does not need to be added. Rectal swabs received fresh or in Cary–Blair medium should be rinsed thoroughly in 1 ml of saline. Before removing the swab, press it against the side of the tube to express any remaining fluid.
2. Add three or more loopfuls of faecal suspension to the enrichment broth.
3. Incubate selenite F broth for 18 hours and APW for 6–8 hours. In some laboratories two or three loopfuls of suspension the initial APW are subcultured in from fresh APW and incubated for another 6–8 hours.
4. Subculture colonies by streaking a loopful of broth on selective and non-selective plating media.

V. cholerae grows quickly in APW and will outgrow other organisms in 6–8 hours. However, after 8 hours other organisms may overgrow the *V. cholerae*. Non-O1 *V. cholerae* grows more quickly than *V. cholerae* O1 and may overgrow the latter when both organisms are present.

Media for enteric pathogens

For *Shigella* spp., *Salmonella* spp. and *Y. enterocolitica* a general-purpose plating medium of low selectivity and a medium of moderate or high selectivity are recommended. MacConkey agar with crystal violet is recommended as a general purpose medium. For *Y. enterocolitica* incubate the MacConkey agar at 35 °C for 1 day and then at room temperature (22–29 °C) for another day.

Xylose–lysine–deoxycholate (XLD) agar is recommended as a medium of moderate or high selectivity for the isolation of *Shigella* and *Salmonella* spp. Deoxycholate citrate agar (DCA), Hektoen enteric agar (HEA) or *Salmonella–Shigella* (SS) agar are suitable alternatives. *Shigella dysenteriae* type 1, *S. sonnei* and enteroinvasive *E. coli* do not grow well on SS agar. However, SS agar may be used for isolating *Y. enterocolitica* when incubated as described for MacConkey agar. Many laboratories include bismuth sulfite agar for isolating *Salmonella typhi* and other species of *Salmonella*.

For *Campylobacter* spp. there are several selective media (Blaser, Butzler, Skirrow) containing different antimicrobial supplements. However, a blood agar base with 5–10% sheep blood containing a combination of a cefalosporin (15 µg/ml), vancomycin (10 µg/ml), amphotericin B (2 µg/ml) and 0.05% ferrows sulfate–sodium metabisulfite–sodium pyruvate (FBP) is acceptable.

For *Vibrio* spp. a selective medium is necessary although many strains may grow on MacConkey agar. Thiosulfate citrate bile salts sucrose (TCBS) agar is selective for *V. cholerae* O1 and non-O1 and for *V. parahaemolyticus*, but it is expensive. Telluride taurocholate gelatine (TTG) agar is another selective medium, but it is not commercially available. Alkaline meat extract agar (MEA) and alkaline bile salt agar (BSA) are inexpensive simple media that can be locally prepared and give excellent results.

Clostridium difficile was difficult to isolate before the development of cefoxitin–cycloserine–fructose agar (CCFA). Formulations using an egg-yolk agar base are preferally used as *C. difficile* is negative for both lecithinase and lipase, while other clostridia commonly found in the gut, such as *C. perfringens*, *C. bifermentans* and *C. sordelli* are lecithinase-positive.

Primary isolation

The specimen should be examined and cultured as soon as it is delivered to the laboratory, as this gives the highest isolation rate of *Shigella* and *Campylobacter* spp. If this is not possible, the specimen should be stored at 4 °C.

A concentrated inoculum of faeces should be used with high selectivity media and a light inoculum of faeces with low selectivity media. In many laboratories, the plates are inoculated directly with the rectal swabs, but care should be taken not to make an inoculation that is overloaded.

Procedure for inoculation of primary isolation media

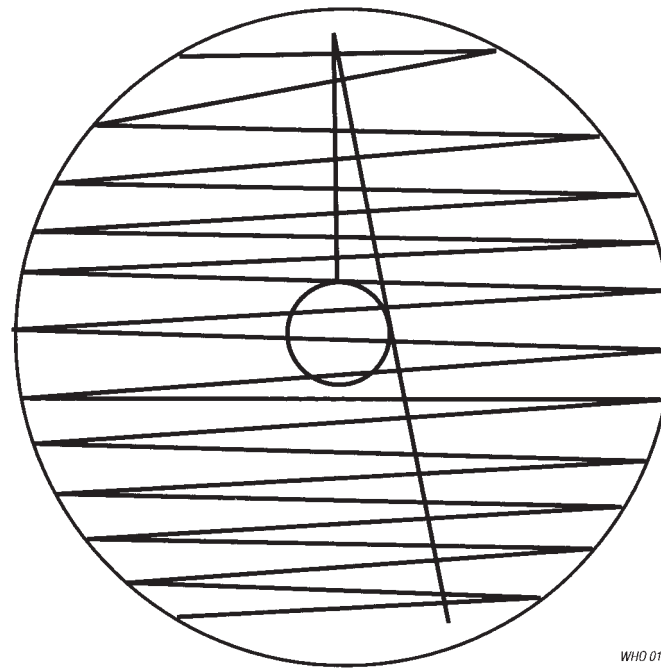
1. Inoculate media of high selectivity with three loopfuls and those of low selectivity with one loopful of the faecal suspension. Place the inoculum in the middle of the agar plate and streak it up and down and across the plate as shown in Fig. 6. This procedure will maximize the number of isolated colonies. Discrete colonies will be found in the peripheral portion of the plate.
2. After inoculation, incubate the agar plates. Incubate the plates for the isolation of *Salmonella, Shigella* and *Yersinia* spp. and *V. cholerae* at 35 °C in an aerobic incubator (without CO_2), the plates for *Campylobacter* spp. at 42 °C in an microaerophilic atmosphere with 10% CO_2, and the plates for *Clostridium difficile* at 35 °C in an anaerobic atmosphere.

Incubation atmosphere for *Campylobacter*

Plates for the isolation of *Campylobacter* spp. should be incubated at 42–43 °C in a microaerophilic atmosphere containing 5% O_2, 10% CO_2 and 85% N_2. The growth of the normal faecal flora is inhibited at this temperature while the thermotolerant *Campylobacter* species are unaffected. However, if non-thermotolerant species are being investigated, the temperature of incubation should be lowered to 35–37 °C.

- An atmosphere suitable for the growth of *Campylobacter* spp. may be produced in several ways. The method of choice will depend on the size and workload of the laboratory, and the relative cost.
- A candle-jar provides an atmosphere of approximately 17–19% O_2 and 2–3% CO_2. This atmosphere is not ideal for the growth of *Campylobacter* spp., and some strains will not grow in it. However, several investigators have demonstrated that incubation at 42 °C on a culture medium supplemented with FBP will improve the isolation rate. The FBP supplement enhances the oxygen tolerance of *Campylobacter* spp. by inactivating super-

Fig. 6. Inoculation of bacteria on culture plates



oxides and hydrogen peroxide. Disadvantages of this system are a longer incubation period and inhibition of some oxygen-sensitive *Campylobacter* spp.

- Another simple and inexpensive system uses a co-culture technique. Plates with rapidly growing facultative anaerobic bacteria are incubated with the plates for the isolation of *Campylobacter* spp. in an airtight container or a plastic bag. As the facultative anaerobic bacteria grow, the oxygen content is lowered and the CO_2 content is increased. The disadvantage of this system is the longer incubation time usually required for growth of the *Campylobacter* spp.
- A hydrogen and CO_2-generator envelope with a self-contained catalyst specifically for the isolation of *Campylobacter* spp. is commercially available. The envelope is placed in an anaerobic jar, and a new envelope must be used each time the jar is opened. No more than six plates should be stacked in the jar to obtain maximum isolation.
- A plastic bag incubation system is also commercially available. It consists of a plastic bag, which is collapsed two or three times by hand or vacuum, and refilled each time, with 5% O_2, 10% CO_2 and 85% N_2.
- The evacuation–replacement system uses an anaerobic jar without a catalyst. The container is evacuated twice to 38 cm (15 mmHg) pressure and refilled each time with 10% H_2 and 90% N_2 mixture.

Preliminary identification of isolates

Identification involves both biochemical and serological tests, the extent of which will depend on the capacity of the laboratory. Flow charts for guidance in identification of important enteric bacteria are presented in Figs 7a–c.

Identify well-separated colonies of typical appearance on the primary plates by making a mark on the bottom of the Petri dish. These will be transferred for further testing. If more than one type of colony is present, process at least one colony of each type.

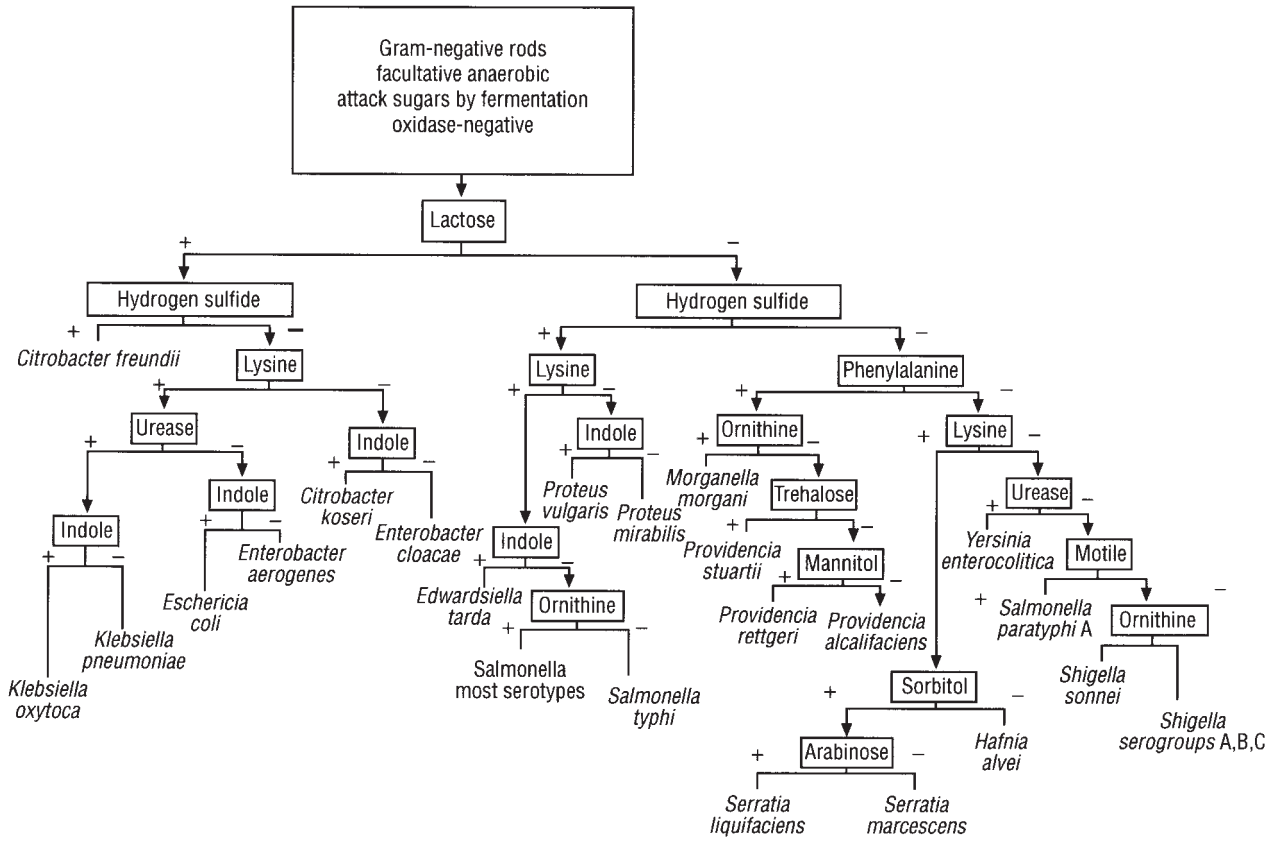
Lactose-nonfermenting bacteria, such as *Salmonella* and *Shigella* spp., give rise to small colourless colonies on MacConkey agar, SS agar, and DCA. Colonies of *Proteus* spp. may be confused with *Salmonella* and *Shigella* spp., especially on MacConkey agar and DCA, because of their lactose-negative appearance. Lactose-fermenting organisms, such as *E. coli* and *Enterobacter/Klebsiella* spp., produce pink to red colonies on MacConkey agar, DCA and SS agar. On XLD agar, *Shigella* and *Salmonella* spp. produce small red colonies, most strains of *Salmonella* with a black centre. Some strains of *Proteus* spp. will also give black-centred colonies on XLD agar. On bismuth sulfite agar, *Salmonella typhi* produces black colonies with a metallic sheen, if the colonies are well separated. *Yersinia enterocolitica* grows on MacConkey and SS agars as small, pale colourless colonies that grow most rapidly at 22–29 °C.

Salmonella and *Shigella* spp.

Three differential media are recommended for initial screening of isolates of *Salmonella* and *Shigella* spp.:

— urea broth, weakly buffered (UREA)
— motility-indole–lysine medium (MIL)
— Kligler's iron agar (KIA).

Fig. 7a. Flow diagram for the preliminary identification of common Enterobacteriaceae



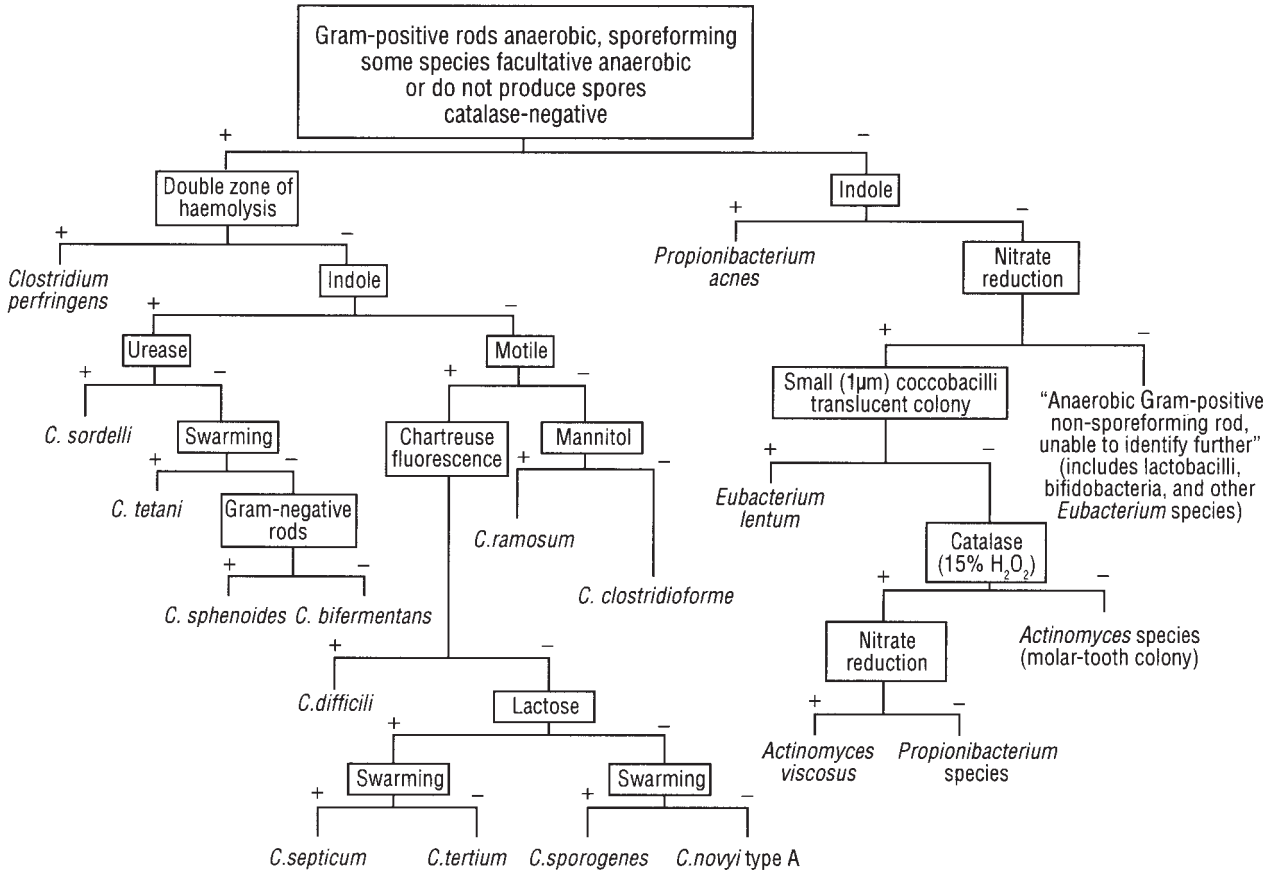
Procedure for inoculation and reading of UREA

1. Using an inoculating loop, collect 2–3 non-lactose-fermenting colonies from the primary plates and transfer to tube containing UREA.
2. Incubate the tubes for 2–4 hours at 35 °C and observe for a change in colour to pink (urease-positive). Discard the urease-positive tubes.
3. Subculture growth from the urease-negative tubes to MIL and to KIA (see below), and incubate all tubes, including the urease-negative tube containing UREA, overnight at 35 °C in an aerobic incubator.

Procedure for inoculation and reading of MIL and KIA

1. Inoculate the MIL by inserting a straight inoculating needle to 2 mm above the bottom of the tube. Withdraw the needle along the same line.
2. Inoculate the KIA by stabbing the agar butt with a straight inoculating needle and streaking the slant in a zigzag.
3. Label all tubes with the number of the laboratory and incubate overnight at 35 °C.
4. Examine the tube of Urease-negative UREA (see above) for delayed urease reaction. Discard the delayed urease-positive cultures.
5. Examine the MIL medium for motility, lysine and indole reaction. Motile organisms will spread out into the medium from the line of inoculation and produce diffuse growth. Non-motile organisms will grow only along the line of inoculation. A positive lysine reaction is indicated by an alkaline reaction (purple colour) at the bottom of the medium, and a negative reaction by an acid reaction (yellow colour) at the bottom of the medium

Fig. 7b. Flow diagram for the preliminary identification of anaerobic Gram-positive rods



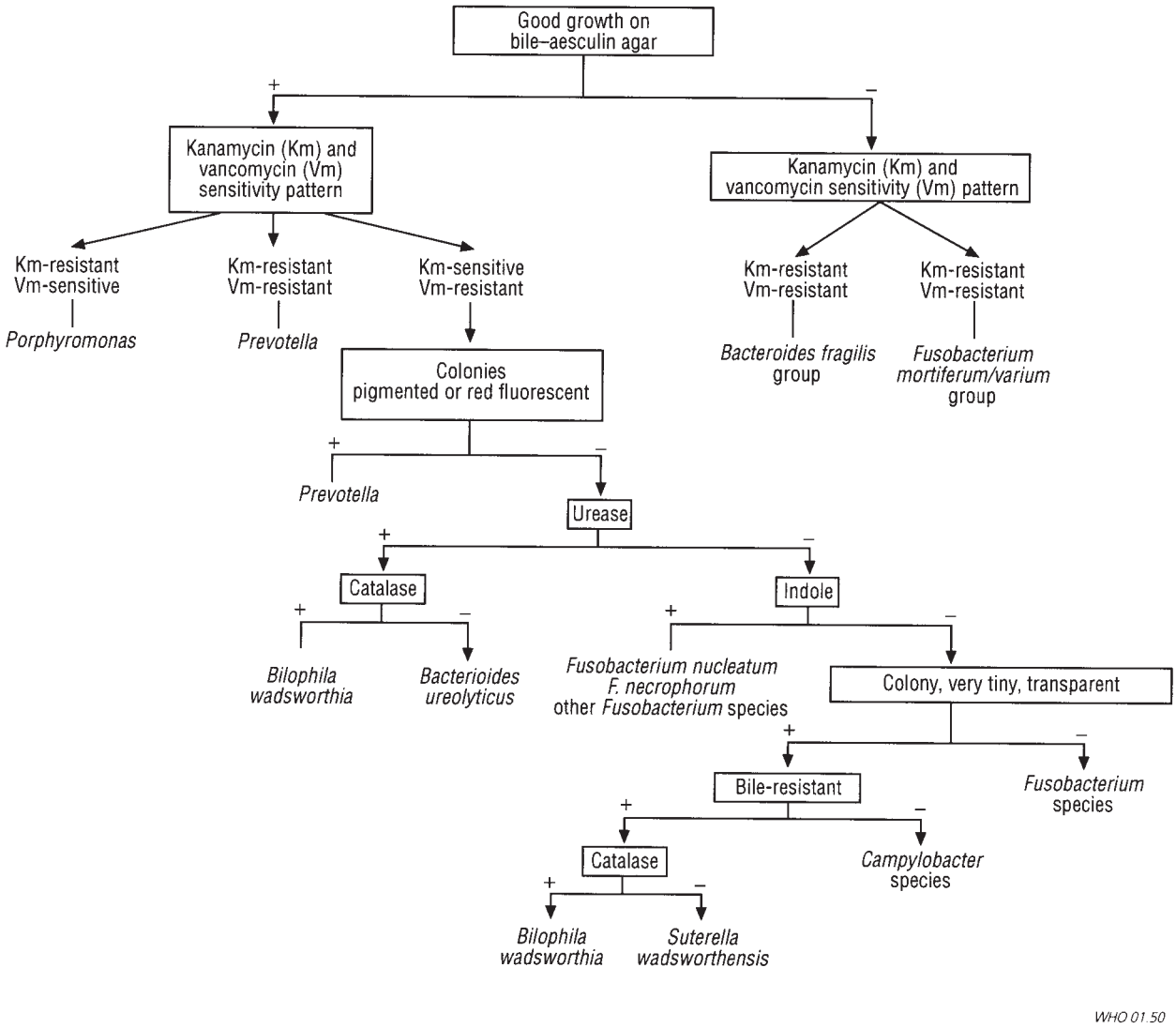
(caused by fermentation of glucose). To test for indole production, add 3–4 drops of Kovacs reagent to the medium. A red to pink colour indicates the presence of indole and the persistence of the bright yellow layer indicates a negative test.

6. Examine the KIA medium. All Enterobacteriaceae ferment glucose, producing acid and gas or acid only, which gives a yellow slant. If gas is produced, bubbles or cracks are seen throughout the medium; the medium may even be pushed up in the tube if a large amount of gas is produced (e.g. in the case of *Enterobacter* spp.). If lactose is simultaneously fermented, both the agar butt and the slant become acid, i.e. yellow (e.g. in the case of *E. coli*). If lactose is not fermented (e.g. in the case of *Shigella* and *Salmonella* spp.), the agar butt is yellow but the slant becomes alkaline, i.e. red. Blackening along the stab line or throughout the medium indicates the production of hydrogen sulfide. Record the result and make a provisional identification of the organism with the help of Tables 10 and 11.

Salmonella strains are oxidase-negative, motile, and indole-negative. They do not hydrolyse urea and—except for *S. paratyphi* A—are lysine-decarboxylase-positive. On KIA agar they produce an alkaline slant, acid butt, H_2S, and gas, except for *S. typhi*, which is anaerogenic, and most strains of *S. paratyphi* A, which are H_2S-negative. If these criteria are satisfied, report: "*Salmonella* isolated (provisional identification)".

Shigella strains are oxidase-negative, non-motile, lysine-decarboxylase-negative, and urea is not hydrolysed. On KIA they produce an alkaline slant and acid butt, no H_2S, and no gas, except for *S. flexneri* serotype 6 (Newcastle and Manchester varieties) and *S. boydii* serotype 14, which are aerogenic.

Fig. 7c. Flow diagram for the preliminary identification of anaerobic Gram-negative rods



Catalase is produced except for *S. dysenteriae* serotype 1, which is catalase-negative. If these criteria are fulfilled, report: "*Shigella* isolated (provisional identification)".

Yersinia enterocolitica

Growth of small, pale or colourless colonies on MacConkey or SS agar after overnight incubation could be *Yersinia enterocolitica*. Inoculate typical colonies into KIA and incubate at 25 °C overnight. Also inoculate suspected colonies into two UREA and two MIL media, incubate 1 tube of each at 25 °C and the others at 35 °C. On KIA, typical *Y. enterocolitica* strains will produce acid butt, alkaline slant, with no gas or H_2S. If the strain is motile and urease-positive at 25 °C and non-motile and weakly urease-positive or urease-negative at 35 °C, report: "*Yersinia* isolated (provisional identification)".

Vibrio cholerae and *V. parahaemolyticus*

Vibrio strains grow as pale, non-lactose-fermenting colonies on MacConkey agar. On TCBS agar *V. cholerae* grows as medium-sized convex, smooth,

Table 9. Colony morphology of common enteric bacteria on differential and selective plating media

Species	MacConkey agar with crystal violet	XLD agar	SS agar	DCA	HEA
Escherichia coli	Pink to rose	Large, flat, yellow, opaque	Pink to red, inhibited, growth	Pink, encircled by zone of precipitate	Large, salmon-pink to orange, encircled by zone of precipitate
Shigella spp.	Colourless	Red	Colourless	Colourless to tan	Green, moist and raised
Salmonella spp.	Colourless	Red, with or without black centre	Colourless, with or without black centre	Colourless to tan, with or without black centre	Blue-green, with or without black centre
Enterobacter/ Klebsiella. spp.	Pink, mucoid	Yellow, mucoid	Pink, inhibited growth	Large, pale mucoid with pink centre	Large, salmon-orange
Proteus/ Providencia spp.	Colourless, inhibited, swarming	Red, some *Proteus* spp. have black centre	Colourless, with or without grey-black centre	Large, colourless to tan, with or without black centre	Blue-green or salmon, with or without black centre
Yersinia enterocolitica	Colourless	Yellow, irregular	Colourless	Colourless	Salmon
Enterococci	No growth	No growth to slight growth	No growth	No growth to slight growth	No growth to slight growth

XLD: Xylose-Lysine–Deoxycholate; DCA: Deoxycholate–citrate; SS: Salmonella-Shigella; HEA: Hektoen enteric

Table 10. Interpretation of Enterobacteriaceae reactions on Kligler's iron agar (KIA)

Reaction	Interpretation
Acid butt (yellow) and alkaline slant (red)	Only glucose fermented
Acid throughout medium (butt and slant yellow)	Glucose and lactose fermented
Alkaline throughout medium (butt and slant red)	Neither glucose nor lactose fermented
Gas bubbles in butt or cracks in the medium	Gas-producing bacteria
Blackening in the butt	Hydrogen sulfide (H_2S) produced

yellow colonies, whereas *V. parahaemolyticus* grows as large, flat, blue-green colonies. Some strains of *V. cholerae* may also appear green or colourless on TCBS because of delayed sucrose fermentation. On TTGA, colonies develop dark centres because of telluride reduction and are surrounded by cloudy zones due to gelatinase activity. On BSA and MEA, *V. cholerae* colonies are translucent, usually with a flat surface and clear-cut margin; they are easily differentiated from colonies of Enterobacteriaceae under oblique light illumination or when examined against the day light at an

Table 11. Typical reaction patterns of Enterobacteriaceae on Kligler's iron agar (KIA)

Reaction	Sugar(s) fermented	Bacteria species
Butt acid Slant acid Gas in butt No H_2S	Glucose: acid and gas Lactose: acid and gas	*Escherichia coli* *Klebsiella* *Enterobacter* *Citrobacter diversus* *Serratia liquefaciens*
Butt acid Slant alkaline Gas in butt H_2S produced	Glucose: acid and gas Lactose: not fermented	*Salmonella* *Proteus* *Citrobacter freundii**
Butt acid Slant alkaline No gas in butt No H_2S	Glucose: acid only Lactose: not fermented	*Shigella* *Yersinia* *Serratia marcescens** *Providencia stuartii* *Providencia rettgeri**
Butt acid Slant alkaline Gas in butt No H_2S	Glucose: acid and gas Lactose: not fermented	*Salmonella paratyphi A* *Hafnia alvei* *Serratia marcescens** *Morganella morgani*
Butt neutral/alkaline Slant alkaline No gas No H_2S	No sugars fermented	*Alcaligenes* *Pseudomonas* *Acinetobacter*

*Atypical reactions.

oblique angle. Suspect colonies should be screened with oxidase and the string test.

Procedure for oxidase test

1. Place 2–3 drops of the oxidase reagent (1% tetramethyl-para-phenylenediamine) on a piece of filter paper in a Petri dish.
2. Pick up a small amount of fresh growth from the MacConkey agar with a platinum (not Nichrome) loop or a clean wooden stick or toothpick. Smear the growth across the moistened part of the filter paper.
3. A positive reaction is indicated by the appearance of a dark purple colour on the paper within 10 seconds. Among the Gram-negative rods *Vibrio*, *Aeromonas*, *Plesiomonas*, *Pseudomonas*, and *Alcaligenes* are oxidase-positive; all Enterobacteriaceae are oxidase-negative. The oxidase reagent should be tested regularly with positive and negative control strains.

Procedure for string test

1. Place a drop of 0.5% aqueous solution of sodium deoxycholate on a slide and mix a small amount of growth from the MacConkey agar into the drop.
2. A positive reaction is indicated by the suspension within 60 seconds: it looses its turbidity and becomes mucoid; a "mucoid string" can be drawn when the loop is slowly lifted away from the drop. A few strains of *Aeromonas* may show a weak and delayed string at about 60 seconds.

If these tests are positive, transfer a part of a colony to KIA and, after overnight incubation, observe for a yellow butt, alkaline slant, and no gas or H_2S production. If this is confirmed, report: "*Vibrio cholerae* isolated (provisional identification)".

Campylobacter jejuni and *Campylobacter coli*

Examine the *Campylobacter* plates after 48–72 hours of incubation. Suspect colonies should be screened with three presumptive tests: oxidase test, wet mount preparation under dark-field or phase-contrast microscope, and Gram-stain. If a dark-field or phase-contrast microscope is not available, colonies may be rapidly screened for typical cell morphology by staining with Gram's crystal-violet solution. For the Gram stain 0.3% carbol fuchsin is recommended as counterstain. *Campylobacter* species are oxidase-positive, they are motile with a darting, tumbling motility, and they appear as simple curved or spiral-shaped rods (seagull wings or "S"-shape). If this is confirmed, report: "*Campylobacter* isolated (provisional identification)".

Clostridium difficile

The *C. difficile* colonies on CCFA are large, yellow and ground glass in appearance. On anaerobic blood agar the colony morphology varies and other features should be looked for to detect the organism. Typically, colonies are grey, opaque and non-haemolytic at 24–48 hours, but a few strains may be greenish blue due to α-type haemolysis. After 48–72 hours of incubation, colonies may develop a distinctly light grey to white centre. With experience, *C. difficile* is easily recognized despite the colonial variability, thanks to its characteristic odour, which resembles that of horse or elephant manure. If the colonies are lecithinase- and lipase-negative and show yellow-green fluorescence when illuminated with Wood's lamp, report: "*Clostridium difficile* isolated (provisional identification)".

Final microbiological identification

Before the final report is made, the cultures should always be checked for pure growth, and the identification should be confirmed with additional biochemical tests.

1. Pick a suspect colony well-separated from other colonies on the plate and subculture it in nutrient broth for biochemical tests, to an agar slope for serological tests, and plate it on MacConkey agar to confirm the purity of the culture.
2. Make additional biochemical tests according to the tables. Examine the reactions after overnight incubation and identify the isolate.

Identification of *Shigella* and *Salmonella* may sometimes pose a problem, because some strains vary in their biochemical reactions and may even share antigens with other Gram-negative organisms. Non-motile, lactose-negative, anaerogenic strains of *E. coli* are notoriously difficult to differentiate from *Shigella*, and identification may be further complicated by the fact that some of these strains may cause bacillary dysentery.

Salmonella

If the results of the preliminary tests are consistent with a *Salmonella* strain, inoculate ornithine decarboxylase, Simmons citrate, ONPG media, and peptone water enriched with mannitol, rhamnose, trehalose, or xylose. Examine the reactions after overnight incubation and identify the isolate according to Table 12. If the results agree with the culture of *Salmonella*, proceed with the serological identification.

Shigella

If the results of the preliminary tests are consistent with a *Shigella* strain, inoculate ornithine decarboxylase, phenylalanine deaminase and ONPG media, and sucrose and xylose peptone water. Examine the reactions after overnight incubation and identify the isolate according to Table 13. If the results agree with the culture of *Shigella*, proceed with the serological identification.

Shigella contains four species, *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*. These are often referred to as subgroups A, B, C, and D, respectively. Some serotypes may provisionally be identified by biochemical reactions and divided into biotypes.

Table 12. Biochemical reactions of *Salmonella* biotypes and other bacteria

	Swarming	H_2S KIA	Indole	Lysine	Ornithine	Citrate	ONPG	Urease	Mannitol	Trehalose	Rhamnose	Xylose
Salmonella (most serotypes)	–	–	–	+	+	+	–	–	+	+	+	+
S. choleraesuis	–	d	–	+	+	d	–	–	+	–	+	+
S. arizonae	–	+	–	+	+	+	+	–	+	+	+	+
S. typhi	–	+w	–	+	–	–	–	–	+	+	–	+
S. paratyphi A	–	d–	–	–	+	–	–	–	+	+	+	–
Edwardsiella tarda	–	+	+	+	+	–	–	–	–	–	–	–
Citrobacter freundii	–	+	–	–	–	+	+	d	+	+	+	+
Proteus spp.	+	+	–/+	–	+/–	v	–	+	–	+	–	+

Abbreviations: +: >95% positive; d: 26–74% positive; d–: 5–25% positive; –: <5% positive; v: variable result; w: weak reactions. H_2S/KIA: hydrogen sulfide production in Kligler's iron agar; Lysine: lysine decarboxylase; Ornithine: ornithine decarboxylase; Citrate: Simmons citrate agar; ONPG: β-galactosidase.

Table 13. Biochemical reactions of *Shigella* biotypes and other bacteria

	Oxidase	Motility	Indole	Lysine	Urease	VP	Citrate	Ornithine	Phenylalanine	ONPG	Sucrose	Xylose
Shigella sonnei	–	–	–	–	–	–	–	+	–	d+	–	–
Shigella, other species	–	–	d	–	–	–	–	–	–	–	–	–
E. coli, inactive strains	–	–	d+	d	–	–	–	d–	–	d	d–	d
Providencia	–	+	+	–	v	–	+	–	+	d–	d	–
Morganella	–	d+	+	–	+	–	–	+	d+	d–	–	–
Hafnia alvei	–	+	–	+	–	d+	d–	+	–	+	d–	+
Serratia marcescens	–	+	–	+	d–	+	+	+	–	+	+	–
Salmonella paratyphi A	–	+	–	–	–	–	–	+	–	–	–	–
Plesiomonas shigelloides	+	+	+	+	–	–	–	+	–	+	–	–

Abbreviations: +: >95% positive; d+: 75–95% positive; d: 26–74% positive; d–: 5–25% positive; –: <5% positive. Lysine: lysine decarboxylase; VP: Voges–Proskauer; Citrate: Simmons citrate agar; Ornithine: ornithine decarboxylase; Phenylalanine: phenylalanine deaminase; ONPG: β-galactosidase.

Table 14. Biochemical reactions of *Shigella* species and serotypes

	Ornithine decarboxylase	Fermentation: lactose/sucrose	Fermentation: mannitol	Catalase	Glucose gas
Shigella dysenteriae					
Serotype 1 (*shigae*)	–	–	–	–	–
Serotype 2 (*schmitzii*)	–	–	–	+	–
Serotypes 3–10	–	–	–	+	–
Shigella flexneri					
Serotype 1–5, X and Y	–	–	+	+	–
Serotype 6 Newcastle	–	–	–	+	+
Serotype 6 Manchester	–	–	+	+	+
Serotype 6 Boyd 88	–	–	+	+	–
Shigella boydii					
Serotype 1–13, 15	–	–	–	–	–
Serotype 14	–	–	–	–	+
Shigella sonnei	+	+(delayed)	+	+	–

S. dysenteriae (subgroup A) contains 10 serotypes. Serotype 1 is catalase-negative and produces Shiga toxin. The other serotypes are catalase-positive. Most strains do not ferment mannitol and lactose.

S. flexneri (subgroup B) contains 8 serotypes. Most strains ferment mannitol but not sucrose or lactose. The Newcastle strain of serotype 6 does not ferment mannitol, but produces gas from glucose; the Manchester strain produces acid and gas from glucose and mannitol; the Boyd 88 strain produces acid but no gas from glucose and mannitol.

S. boydii (subgroup C) contains 15 serotypes. Mannitol is fermented, but lactose is not fermented.

S. sonnei (subgroup D) contains one serotype with two "phases": I and II. Mannitol is fermented. ONPG is positive, but fermentation of lactose and sucrose is delayed until after 24 hours. (See Table 14.)

Yersinia enterocolitica

If the results of the preliminary tests are consistent with a *Yersinia* strain, inoculate ornithine decarboxylase, Voges–Proskauer, ONPG, Simmons citrate agar, and peptone water enriched with sucrose, rhamnose, mellibiose, sorbitol or cellobiose. Examine the reactions after overnight incubation and identify the isolate according to Table 15. If the results agree with the culture of *Y. enterocolitica*, report: "*Yersinia enterocolitica*".

Vibrio cholerae

If the results of the preliminary tests are consistent with a *Vibrio* strain, inoculate ornithine decarboxylase, Simmons citrate agar, and sucrose peptone water, and incubate overnight. If one of these reactions is negative, test for aesculin hydrolysis, Voges–Proskauer, and fermentation of mannitol, arabinose and arbutin. Examine the reactions after overnight incubation and identify the isolate according to Table 16.

Table 15. Biochemical reactions of *Yersinia enterocolitica* and other non-pathogenic *Yersinia* species

	MIL	Urease	Ornithine	VP 25 °C	Citrate	Sucrose	Rhamnose	Mellibiose	Sorbitol	Cellobiose
Y. enterocolitica	+/v/–	+	+	v	–	v	–	–	+*	+
Y. frederiksenii	+/+/–	+	+	+	d	+	+	–	+	+
Y. intermedia	+/+/–	+	+	+	+	+	+	+	+	+
Y. kristensenii	+/d/–	+	+	–	–	–	–	–	+	+
Y. pseudotuberculosis	+/–/–	+	–	–	–	–	+	+	–	–

Abbreviations: +: >95% positive; d: 26–74% positive; –: <5% positive; v: variable result; MIL: motility–indole–lysine medium; VP 25 °C: Voges–Proskauer agar at 25 °C incubation; Citrate: Simmons citrate agar.
*except biotype 5.

Table 16. Biochemical reactions of vibrios found in stool

	Oxidase	KIA butt/slant	MIL	Ornithine	Citrate	Sucrose	Mannitol	Arabinose	Aesculin	Comments
Vibrio cholerae	+	K/A	+/+/+	+	+	+	+	–	–	
V. mimicus	+	v/A	+/+/+	+	+	–	+	–	–	
V. parahaemolyticus	+	K/A	+/+/+	+	–	–	+	d+	–	
V. fluvialis	+	K/A	+/d/–	–	+	+	+	+	–	
V. furnissii	+	K/AG	+/–/–	–	+	+	+	+	–	
V. hollisae	+	K/A	+/+/–	–	–	–	–	+	–	Poor grower
Aeromonas hydrophila	+	A/AG	+/+/+	–	d	+	+	+	+	Arb +/VP +
A. caviae	+	A/A	+/+/–	–	+	+	+	+	d	Arb –/VP –
A. veronii biotype *sobria*	+	A/AG	+/+/+	–	+	+	+	–	–	Arb –/VP +
Plesiomonas shigelloides	+	K/A	+/+/+	+	–	–	–	–	+	Arb –/VP –

Abbreviations: +: >95% positive; d+: 75–95% positive; d: 26–74% positive; –: <5% positive; KIA: Kligler's iron agar; MIL: motility–indole–lysine medium; Ornithine: ornithine decarboxylase; Aesculin: aesculin hydrolysis; K: alkaline; A: acid; G: gas; Arb: arbutin fermentation; VP: Voges–Proskauer.

Table 17. Differentiation of biotypes of *Vibrio cholerae*

	Biotype	
	Classical	El Tor
Haemagglutination	Negative reaction, no growth*	Positive reaction, growth*
Polymyxin B (50 units)	Sensitive	Resistant
Voges–Proskauer	Negative reaction, no growth*	Positive reaction, growth*
Haemolysis	Negative reaction, no growth	Variable

*Aberrant reactions may occur.

If a specific anti-*Vibrio cholerae* serogroup O1 serum is available, make a rapid slide agglutination test. In case of macroscopic agglutination report: "*Vibrio cholerae* O1". If the antiserum is not available or if the identification is uncertain, send the isolate to a reference laboratory.

Differentiation of *V. cholerae* O1 into classical and El Tor biotypes is not necessary for treatment or control, but for some of the isolates it should be done by one of the following tests (Table 17).

The indirect haemagglutination test

1. Prepare a 2.5% suspension of chicken or sheep red blood cells by repeated centrifugation and dilution in saline.

2. Divide a clean glass slide into several squares with a pencil and place a loopful (3 mm) of the red cell suspension in each square.
3. Place a small portion of the growth from an agar or KIA slant in each red cell suspension and mix well.

Clumping of the red cells occurs within 30–60 seconds with strains of the El Tor biotype. Known haemagglutinating (El Tor) and non-haemagglutinating strains should be used as controls for each new suspension of red cells. Newly isolated strains of classical biotypes are usually negative in the test, but old laboratory strains of the classical biotype may not always be negative in this reaction.

Polymyxin B susceptibility test

1. Spread a loopful of overnight peptone water culture of the isolate on a Mueller–Hinton or meat-extract agar.
2. Place a susceptibility disk containing 50 units of polymyxin B in the middle of the culture.
3. Place the plate in the refrigerator for 1 hour.
4. Incubate the plate overnight at 35 °C.

Known strains of classical and El Tor biotypes should always be included as controls. Classical strains are sensitive to polymyxin B and a clear inhibitory zone is observed around the disk. The El Tor strains are resistant and no inhibitory zone is formed.

Campylobacter jejuni and *Campylobacter coli*

Only a few tests are available in clinical laboratories to identify the *Campylobacter* species and subspecies. *Campylobacter* species are usually divided into two groups on the basis of growth temperature: thermotolerant species, which grow at 42–43 °C, and non-thermotolerant species, which grow at 15–25 °C.

The thermotolerant species are *Campylobacter jejuni* subsp. *jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and some strains of *C. hyointestinalis*. *C. lari* and *C. hyointestinalis* are resistant to nalidixic acid; *C. jejuni*, *C. coli* and *C. upsaliensis* are sensitive to it. *C. jejuni* subsp. *jejuni*, *C. coli* and *C. lari* are resistant to cefalotin; *C. hyointestinalis* and *C. upsaliensis* are sensitive to it. The differentiation within these groups is made on the basis of hippurate hydrolysis and the production of hydrogen sulfide in Kligler's iron agar.

The non-thermotolerant species are *C. jejuni* subsp. *doylei*, *C. fetus*, and *Arcobacter butzleri*. *C. jejuni* subsp. *doylei* will grow at neither 15 °C nor at 25 °C; *C. fetus* will grow at 25 °C but not at 15 °C; *A. butzleri* will grow at both temperatures. *A. butzleri* is resistant to cefalotin; *C. jejuni* subsp. *doylei* and *C. fetus* are sensitive to it (Table 18).

Serological identification

Salmonella

The nomenclature and classification of the salmonellae have changed several times and are still under discussion. According to present nomenclature, all *Salmonella* species belong to a single genus which is subdivided in six subgroups (also called subspecies), including the former genus *Arizona*. Subgroup

Table 18. Biochemical indentification of *Campylobacter* species found in stool

	Growth at			H_2S/KIA	Hippurate hydrolysis[a]	Nitrate reduction	Susceptible to	
	15 °C	25 °C	42 °C				Nalidixic acid[b]	Cefalotin[c]
Campylobacter jejuni subsp. *jejuni*	–	–	+	–	+	+	S	R
C. jejuni subsp. *doylei*	–	–	–	–	d	–	S	S
C. coli	–	–	+	+	–	+	S	R
C. lari	–	–	+	–	–	+	R	R
C. upsaliensis	–	–	+	–	–	+	S	S
C. fetus subsp. *fetus*	–	+	–	–	–	+	R	S
C. hyointestinalis	–	+	v	+	–	+	R	S
Arcobacter butzleri[d]	+	+	–	–	–	+	v	R

Abbreviations: +: >95% positive; d+: 75–95% positive; d: 26–74% positive; d–: 5–25% positive; –: <5% positive; v: variable result; S: susceptible; R: resistant. H_2S/KIA: Kligler's iron agar.

[a]Only deep purple colour is considered positive.

[b]30 µg nalidixic acid disk.

[c]30 µg cefalotin disk.

[d]Catalase-negative or catalase weakly positive; Kligler's iron agar.

1 corresponds to the typical salmonellae and includes among others: *Salmonella typhi*, *S. paratyphi* A, *S. enteritidis*, *S. typhimurium*, *S. choleraesuis*. This subgroup contains more than 2000 serotypes, which can be differentiated by their antigenic formula (O, H and Vi antigens). Serotypes in subgroup 1 continue to be named as if they were real species: *Salmonella* subgroup 1 serotype *typhimurium* is simply called *S. typhimurium*. More than 99% of human salmonella isolates belong to subgroup 1.

The important antigens for serotyping *Salmonella* species are the somatic, or O, antigens and the flagellar, or H, antigens. O antigens are present in both motile and nonmotile organisms and are resistant to boiling; H antigens are present only in motile organisms and are sensitive to boiling. The majority of the *Salmonella* species are diphasic in the motile state and may exhibit two antigenic forms referred to as phases 1 and 2. These phases share the same O antigens but possess different H antigens. To identify the serotype it is necessary to identify the specific H antigens in both phases. These may not always be evident, and phase suppression may be necessary to confirm the latent phase.

O antigens are designated by Arabic numerals. Phase 1 H antigens are designated by small Roman letters and phase 2 H antigens by Arabic numerals. For example, the antigenic formula for *S. typhimurium* is 1,4,[5],12:i:1,2, where O antigens are 1, 4, 5 and 12; phase 1 of the H antigen is "i" and phase 2 antigens are 1 and 2. A bracket indicates that the antigen may be absent, and an underlined antigen indicates that the antigen is associated with lysogenic conversion by a bacteriophage. This change in antigenic structure is only present when the bacteriophage is present and may be the only difference between certain serotypes.

The *Salmonella* species have been placed in groups according to the presence of certain O antigens. These groups are often referred to as the Kauffmann–White scheme. Capital Roman letters designate the O group. In the original scheme the groups were A, B, C, D and E; these were subsequently expanded to A–Z with 4 subgroups in C, 3 in D, 4 in E and 2 in G. The O groups are defined by the presence of certain O antigens as follows:

Group:	A	B[a]	C_1	C_2	D	E_1	F
Antigen:	2	4;5	6;7	6;8	9	3;10	11

There are other variations in the antigenic structure: the change in colony appearance from smooth to rough, and the presence or absence of Vi antigen. When present the Vi antigen prevents agglutination by homologous O antiserum. The Vi antigen is usually found in fresh isolates and is lost rapidly on storage of the culture. The Vi antigen is important for the identification of *S. typhi*, which often is not agglutinated by heterologous H antisera.

Procedure for somatic O antigen analysis

Direct slide agglutination test

1. Bring the saline and the reagents to room temperature before performing the test.
2. Place a drop of saline on a clean microscope slide.
3. With a sterile loop, emulsify a small portion of the growth from a moist agar slope in the drop of saline to produce a uniform and turbid suspension.
4. Examine the bacterial suspensions through a hand-lens or the low-power objective (×10) of a microscope to ascertain that the suspension is not auto-agglutinable in saline.
5. With a 10-µl loop take up 1 drop of *Salmonella* polyvalent O antiserum (A–I and Vi), and place it on the slide just beside the bacterial suspension.
6. Mix the antiserum and bacterial suspension and tilt the slide backwards and forwards for 1 minute. Look for clumping while viewing the suspension under good light. Distinct clumping during this period is a positive result.
7. If the result is positive, repeat the slide test with single-factor antiserum.

Some *Salmonella* possess an envelope (Vi) antigen and, in the live or unheated form, are non-agglutinable with group C1 (O:6,7) or group D (O:9) antisera. Heat the suspension in boiling water for 20 minutes to remove the Vi antigen, cool, separate the bacteria by centrifugation, re-suspend in fresh saline, and test with the same antisera.

Procedure for H antigen analysis

A preliminary identification of the major flagellar (H) antigen can be made by the direct slide agglutination test as described for the somatic O antigens. Occasionally it is necessary to increase the motility of the test organism by making several consecutive transfers in a semi-solid nutrient medium ("swarm agar", see below). Antisera against those antigens are commercially available. However, a phase suppression is often required if identification of both flagellar phases is necessary for classification. This often requires a phase inversion with sera designed for this purpose.[1]

1. Prepare a semi-solid nutrient medium containing 0.2–0.4% agar. Add 1 ml of the medium to test-tubes.
2. Replace the corks from the test-tubes with cotton wool.
3. Melt the agar in boiling water and place the test-tubes with the melted agar in a 45 °C water-bath for 30 minutes.

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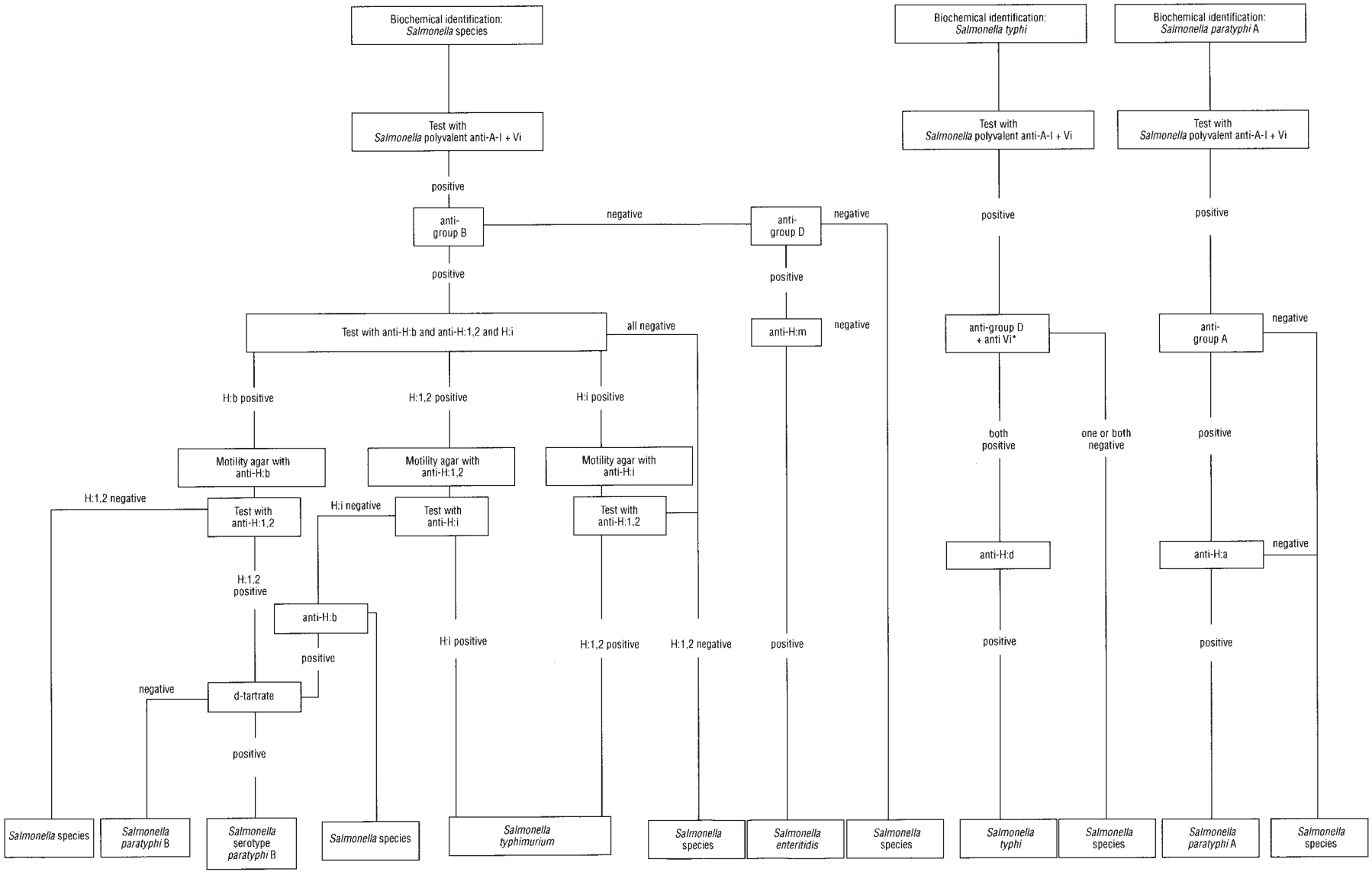
[a] All Salmonellae of subgroup B contain antigen 4, but only some contain antigen 5.
[1] Available from Statens Serum Institut, 5 Artillerivej, 2300 Copenhagen S, Denmark.

Fig. 8. Serological identification of *Salmonella* species



*If only anti-Vi gives agglutination with the organism, boil the bacterial suspension and repeat the test with antiserum O:D

WHO 01.51

4. Write the specimen number and the H antisera (H:b, H:i, and H:1,2) on each test-tube. Include a control test-tube.
5. Add 10µl of each phase inversion heterologous H antiserum to the corresponding agar, shake the test-tube carefully, and leave the agar to solidify as a slope.
6. Make a heavy suspension of isolated colonies in saline, streak the slope using a loop, and incubate overnight.
7. With an inoculating loop emulsify a speck of the culture from the slope in a drop of saline.
8. With a 10-µl loop, take up 1 drop of one of the heterologous H antisera and place it on the slide just beside the bacterial suspension.
9. Mix the antiserum and bacterial suspension and tilt the slide backwards and forwards for 1 minute. Look for clumping while viewing the suspension under a good light. Distinct clumping during this period is a positive result.
10. Repeat the agglutination test with the other heterologous antisera if required, and identify the serotype with the help of the flow diagram on page 57.

Salmonella typhi

To confirm the biochemical identification, test in antisera Vi, O group D (O:D), and H:d. Cultures agglutinating in Vi may be negative in O:D due to obstruction by the Vi antigen. Remove the Vi antigen by heating the suspension for 20 minutes at 100 °C and test in O:D antiserum again. If positive in Vi, O:D and H:d, report: "*Salmonella typhi*". If positive only in O:D, report: "*Salmonella*, group D".

Salmonella paratyphi A

To confirm the biochemical identification, test with *Salmonella* group A antiserum. If positive with group A antiserum, test with H:a antiserum, and if positive, report: "*Salmonella paratyphi* A". If negative with H:a, report: "*Salmonella* group A". If non-motile, they may be *S. flexneri* type 6 or *S. boydii* type 13 or 14, and should be tested with *Shigella* antisera B and D.

Salmonella, other serotypes

When the presumptive tests indicate typical *Salmonella*, test with *Salmonella* O groups A, B, C, D, and E antisera.

- If positive in group B, test with flagellar antiserum H:b, H:i and H:1,2. If positive with H:b antiserum, the organism may be *S. wien* or *S. paratyphi* B. *S. wien* can be differentiated from *S. paratyphi* B by testing with H:1,w and H:2 antisera (if available). *S. wien* reacts with H:1,w and *S. paratyphi* B with H:2.
- If positive with H:i or H:1,2 antiserum, make a phase inversion and test with heterologous H antiserum. If positive, report: "*S. typhimurium*". If negative, report: "*Salmonella*, group B".
- If positive with group C antiserum, report: "*Salmonella* group C".
- If positive with group D antiserum, test with Vi, H:d and H:m antisera. If positive with Vi or H:d, report: "*Salmonella typhi*". If positive with H:m, report: "*Salmonella enteritidis*". If negative with Vi, H:d and H:m, report: "*Salmonella* group D".
- If biochemically the strain is a *Salmonella* but is negative with all O-group antisera, report: "Presumptive *Salmonella* species" and refer to National Reference Centre.

Shigella

Shigella can be subdivided into serogroups and serotypes by slide and tube agglutination tests with specific O-antisera. Slide agglutination will usually be sufficient, if the results are clear-cut. The antigen suspension should be made from a non-selective medium such as a nutrient agar or KIA, and the suspension should be observed for auto-agglutination before the antiserum is added.

Tests with Shigella *groups A, B, C, and D antisera*

- If agglutination occurs with group A, report: "*Shigella dysenteriae*". Test with *S. dysenteriae* type 1 antiserum. If positive, report: "*S. dysenteriae* type 1".
- If agglutination occurs with group B, report: "*Shigella flexneri*".
- If agglutination occurs with group C, report: "*Shigella boydii*".
- If agglutination occurs with group D, report: "*Shigella sonnei*".

Occasionally *Shigella* strains may fail to agglutinate in homologous antiserum due to the presence of a K antigen. Heating a saline suspension of the strain in a boiling water-bath for 20 minutes and repeating the test may reverse this.

Other Gram-negative organisms may share antigens with *Shigella* strains and give false-positive agglutination with *Shigella* typing sera. Well-known examples of bacteria showing this cross-reaction are *Plesiomonas shigelloides* and *Shigella sonnei* phase 1, and certain strains of *Hafnia* and *Shigella flexneri* serotype 4a; but of greater importance is the cross-reaction with some strains of diarrhoeagenic *E. coli*.

Yersinia enterocolitica

Y. enterocolitica possesses several somatic (O) antigens, which have been used for subdividing the species into at least 17 serogroups. Most human infections in Canada, Europe, and Japan are due to serotype O3; Infections due to serotype O9 have been reported mainly from the Scandinavian countries, and infections with serotype O8 are almost exclusively from the USA. There is serological cross-reaction between *Y. enterocolitica* O9 and *Brucella* spp.

Upper respiratory tract infections

Introduction

The upper respiratory tract extends from the larynx to the nostrils and comprises the oropharynx and the nasopharynx together with the communicating cavities, the sinuses and the middle ear. The upper respiratory tract can be the site of several types of infection:

— pharyngitis, sometimes involving tonsillitis, and giving rise to a "sore throat"
— nasopharyngitis
— otitis media
— sinusitis
— epiglottitis.

Of all those infections, pharyngitis is by far the most frequent; in addition, the untreated infection may have serious sequelae. Only pharyngitis will be considered here.

Most cases of pharyngitis have a viral etiology and follow a self-limiting course. However, approximately 20% are caused by bacteria and usually require treatment with appropriate antibiotics. As the physician is rarely able to make a distinction between viral and bacterial pharyngitis on clinical grounds alone, treatment should ideally be based on the result of bacteriological examination.

Bacteriological diagnosis of pharyngitis is complicated by the fact that the oropharynx contains a heavy, mixed, normal flora of aerobic and anaerobic bacteria. The normal flora generally outnumbers the pathogens and the role of the bacteriologist is to distinguish between the commensals and the pathogens. Where possible only the latter should be reported to the physician.

Normal flora of the pharynx

The normal flora of the pharynx includes a large number of species that should be neither fully identified nor reported when observed in throat cultures:

- viridans (α-haemolytic) streptococci and pneumococci
- nonpathogenic *Neisseria* spp.
- *Moraxella* (formerly *Branhamella*) *catarrhalis* (this can also be a respiratory pathogen)
- staphylococci (*S. aureus*, *S. epidermidis*)
- diphtheroids (with the exception of *C. diphtheriae*)
- *Haemophilus* spp.
- yeasts (*Candida* spp.) in limited quantity
- various strictly anaerobic Gram-positive cocci and Gram-negative rods, spirochaetes and filamentous forms.

The throats of elderly, immunodeficient, or malnourished patients, particularly when they have received antibiotics, may be colonized by Enterobacteriaceae (*Escherichia coli*, *Klebsiella* spp., etc.) and by the nonfermentative Gram-negative groups (*Acinetobacter* spp. and *Pseudomonas* spp.). Such patients may also have in their pharynx a proliferation of *S. aureus* or of

Candida spp., or other yeast-like fungi. Although these microorganisms do not cause pharyngitis, except in association with granulocytopenia, it is advisable to report such isolates to the clinician, as they occasionally indicate the existence of (or may sometimes give rise to) a lower respiratory tract infection (e.g. pneumonia) or bacteraemia. However, an antibiogram should not be performed routinely on these colonizing microorganisms.

Bacterial agents of pharyngitis

Streptococcus pyogenes (Lancefield group A) is by far the most frequent cause of bacterial pharyngitis and tonsillitis. This infection is particularly prevalent in young children (5–12 years). When streptococcal pharyngitis is associated with a characteristic skin rash, the patient is said to have scarlet fever. In infants, a streptococcal throat infection may often involve the nasopharynx and be accompanied by a purulent nasal discharge.

Non-group-A, β-haemolytic streptococci (e.g. groups B, C and G) are uncommon causes of bacterial pharyngitis and if detected should be reported. Pharyngeal infections due to *S. pyogenes*, if not properly treated, may give rise to sequelae such as rheumatic fever, and, less often, glomerulonephritis. Specific identification of, and antibacterial treatment directed against, *S. pyogenes* are primarily intended to prevent the occurrence of rheumatic fever.

Corynebacterium diphtheriae is the cause of diphtheria, a disease that is endemic in many countries. It can reach epidemic proportions in countries where the vaccination programme has been interrupted. Characteristically (with a few exceptions), *C. diphtheriae* causes a typical form of infection, characterized by a greyish-white membrane at the site of infection (pharynx, tonsils, nose, or larynx). Diphtheria is a serious disease and the diagnosis is made on the basis of clinical findings. The physician would then generally make a specific request to culture for diphtheria bacilli.

Gonococcal pharyngitis has been recognized with increasing frequency in some countries, with rates that parallel the incidence of cervical and urethral gonorrhoea. Culture of throat swabs for gonococci should be done on specific request from the clinician, using the appropriate selective medium (modified Thayer–Martin medium).

Necrotizing ulcerative pharyngitis (Vincent angina) is a rare condition characterized by a necrotic ulceration of the pharynx with or without formation of a pseudomembrane. It is associated, at the site of infection, with a heavy mixed flora of strict anaerobes dominated by Gram-negative fusiform rods and spirochaetes, generally referred to as *Fusobacterium* spp. and *Treponema vincentii*, and possibly others. Although both species belong to the normal mouth flora, their presence in large numbers in a Gram-stained smear of ulcerated lesions should be reported as a "fusospirochaetal complex". This microscopic diagnosis need not be confirmed by anaerobic culture, which is difficult and time-consuming. However, the presence of this complex does not exclude the need to search for other pathogens, particularly *S. pyogenes.*

Although small numbers of *C. albicans* or other *Candida* species may be part of the normal oral flora, oral candidiasis results when the number of organisms increases considerably in certain pathological conditions, e.g. in malnourished premature babies, in immunodeficient adults (e.g. patients with HIV/AIDS), or in patients who have received broad-spectrum antimicrobials or cancer therapy. The affected area—tongue, tonsils, throat or buccal mucosa—may be extremely red, or covered with white patches or a confluent grey-

white membrane (thrush). The diagnosis of candidiasis is best made by finding numerous yeast cells, some of them forming long mycelium-like filaments, in a Gram-stained smear of the exudate.

Swabs from the upper respiratory tract may be submitted to the laboratory, not for the diagnosis of a clinical infection, but to detect a potential pathogen in a healthy subject, a pharyngeal or a nasal "carrier". This should only be done as part of well-defined epidemiological surveys. The following pathogens can give rise to a carrier state in the upper respiratory tract:

- *Staphylococcus aureus*. Sampling of patients and staff for nasal carriers is sometimes performed as part of an investigation of hospital outbreaks of Meticillin-resistant *S. aureus* (MRSA).
- *Neisseria meningitidis*. Carriage of meningococci may be very prevalent (20% or more) even at non-epidemic times. Identification of pharyngeal carriers of meningococci is rarely needed, and need not be performed prior to the administration of prophylactic antibiotics to family or other close contacts of patients with meningococcal disease.
- *Streptococcus pyogenes*. Carriage of this organism in low numbers may be prevalent, especially among schoolchildren (20–30%).
- *Corynebacterium diphtheriae*. The carrier rate of the diphtheria bacillus is high in non-vaccinated populations. In such communities, it may be justified to identify and treat carriers among the close contacts of a patient with proven diphtheria. Carriers are rare when an immunization programme is correctly implemented.

Collection and dispatch of specimens

Ideally, specimens should be collected by a physician or other trained personnel. The patient should sit facing a light source. While the tongue is kept down with a tongue depressor, a sterile cotton-wool swab is rubbed vigorously over each tonsil, over the back wall of the pharynx, and over any other inflamed area. Care should be taken not to touch the tongue or buccal surfaces. It is preferable to take two swabs from the same areas. One can be used to prepare a smear, while the other is placed into a glass or plastic container and sent to the laboratory. Alternatively, both swabs may be placed in the container and dispatched to the laboratory. If the specimen cannot be processed within 4 hours, the swab should be placed in a transport medium (e.g. Amies or Stuart).

Direct microscopy

The fusospirochaetal complex of necrotizing ulcerative pharyngitis (Vincent angina) and *Candida* are best recognized on a Gram-stained smear, which should be prepared if the physician makes a special request. The Gram-stained smear is not useful for the detection of streptococci or *Neisseria* spp. Moreover, the direct smear has poor sensitivity and specificity for the detection of the diphtheria bacillus, unless the specimen has been collected with care and is examined by an experienced microbiologist. In the absence of a physician's request or of clinical information, a Gram-stained smear should not be made for throat swabs.

Culture and identification

Culture for *Streptococcus pyogenes*

Immediately upon receipt in the laboratory, the swab should be rubbed over one-quarter of a blood agar plate, and the rest of the plate streaked with a sterile wire loop. The blood agar should be prepared from a basal agar medium without glucose (or with a low glucose content), e.g. tryptic soy agar (TSA). Acidification of glucose by *S. pyogenes* inhibits the production of haemolysin. Blood from any species, even human blood (fresh donor blood), can be used at a concentration of 5%. The plates should be filled to a depth of 4–5 mm. Sheep blood is preferred because it does indicate haemolysis of some commensal *Haemophilus* spp. and it gives no haemolysis with the *zymogenes* variant of *Enterococcus faecalis.*

The recognition of β-haemolytic colonies can be improved, and their presumptive identification hastened, by placing a co-trimoxazole disc (as used for the susceptibility test) and a special low-concentration bacitracin disc over the initial streaked area. Because *S. pyogenes* is resistant and many other bacteria are susceptible to co-trimoxazole, this disc improves the visibility of β-haemolysis. Incubation in a candle-jar will detect most β-haemolytic streptococci. A simple way to increase haemolysis is to stab the agar surface perpendicularly by inserting the loop deep into the medium to encourage growth of subsurface colonies. After 18 hours and again after 48 hours of incubation at 35–37 °C, the blood plates should be examined for the presence of small (0.5–2 mm) colonies surrounded by a relatively wide zone of clear haemolysis. After Gram-staining to verify that they are Gram-positive cocci, the colonies should be submitted to specific identification tests for *S. pyogenes.* For clinical purposes, presumptive identification of *S. pyogenes* is based on its susceptibility to a low concentration of bacitracin. For this purpose, a special differential disc is used containing 0.02–0.05 IU of bacitracin. The ordinary discs used in the susceptibility test, with a content of 10 units, are not suitable for identification. A β-haemolytic streptococcus showing any zone of inhibition around the disc should be reported as *S. pyogenes*. If the haemolytic colonies are sufficiently numerous, the presence or absence of an inhibition zone may be read directly from the primary blood agar plate. If the colonies are less numerous, one or two should be picked from the primary plate, streaked on one-fifth of another plate to obtain confluent growth, and each inoculated area covered with a bacitracin disc. After overnight incubation, the subcultures should be read for inhibition zones.

In some laboratories this presumptive identification is confirmed by serological demonstration of the specific cell wall polysaccharides. This can be done either by the classical precipitin method, or more rapidly by using a commercial kit for the rapid slide coagglutination or latex agglutination tests. If desirable, bacitracin-resistant β-haemolytic streptococci can be further identified using some simple physiological tests (see Table 19). Minute colonies of β-haemolytic streptococci may be encountered, which, when grown and serologically grouped, react with group A antiserum. These streptococci are not considered to be *S. pyogenes* and are not associated with the serious infections caused by group A streptococci.

In reporting the presence of *S. pyogenes* in a throat culture, a semiquantitative answer should be given (rare, +, + +, or + + +). Patients with streptococcal pharyngitis generally show massive growth of *S. pyogenes*, with colonies over the entire surface of the plate. Plates of carriers generally show fewer than 20 colonies per plate. Even rare colonies of β-haemolytic streptococci should be confirmed and reported.

Table 19. Differentiation of β-haemolytic streptococci

Species	*S. pyogenes*	*S. agalactiae*	*E. faecalis* var. *zymogenes*[a]	Others
Lancefield group	**A**	**B**	**D**	**C, G, F**
Haemolysis	β	β[b]	β	β
Zone around the differential bacitracin disc	+	0[c]	0[c]	0[d]
Bile–aesculin agar (growth & blackening)	0	0	+	0
Reverse CAMP test	0	+	0	0
Co-trimoxazole[e] susceptibility	0	0	0	+
PYR test[f]	+	0	+	0

[a]*E. faecalis* var. *zymogenes* produces β-haemolysis only on horse-blood agar.
[b]5% are non-haemolytic.
[c]5% are positive.
[d]10% are positve.
[e]Same disc as in the Kirby–Bauer method.
[f]PYR: pyrrolidonyl-β-naphtylamide.

Culture for *Corynebacterium diphtheriae*

Although the diphtheria bacillus grows well on ordinary blood agar, growth is improved by inoculating one or two special media:

- *Löffler coagulated serum or Dorset egg medium.* Although not selective, both of these media give abundant growth of the diphtheria bacillus after overnight incubation. Moreover, the cellular morphology of the bacilli is more "typical": irregularly stained, short to long, slightly curved rods, showing metachromatic granules, and arranged in a V form or in parallel palisades. Metachromatic granules are more apparent after staining with methylene blue or Albert stain than with the Gram stain.
- *A selective tellurite blood agar.* This medium facilitates isolation when the bacilli are few in number, as is the case for healthy carriers. On this medium, colonies of the diphtheria bacillus are greyish to black and are fully developed only after 48 hours. Suspicious colonies, consisting of bacilli with a coryneform morphology on the Gram-stained smear, should be subcultured to a blood agar plate to check for purity and for "typical" morphology. It should also be remembered that colonies of the *mitis* biotype of *C. diphtheriae*, which is the most prevalent, show a marked zone of β-haemolysis on blood agar.

A presumptive report on the presence of *C. diphtheriae* can often be given at this stage. However, this should be confirmed or ruled out by some simple biochemical tests and by demonstration of the toxigenicity. As the latter requires inoculation of guinea-pigs or an in vitro toxigenic test (Elek) and has to be performed in a central laboratory, only rapid biochemical identification will be covered here. *C. diphtheriae* is catalase- and nitrate-positive. Urea is not hydrolysed. Acid without gas is produced from glucose and maltose, generally not from saccharose. The fermentation of glucose can be tested on Kligler medium. Urease activity can be demonstrated on MIU and nitrate reduction in nitrate broth in the same way as for Enterobacteriaceae. For the fermentation of maltose and saccharose, Andrade peptone water can be used as a base with a 1% final concentration of each carbohydrate. Results can usually be read after 24 hours, although it may be necessary to reincubate for one night.

It must be emphasized that the microbiology laboratory's role is to confirm the clinical diagnosis of diphtheria. Therapy should not be withheld pending receipt of laboratory reports. More detailed information on the isolation and identification of *C. diphtheriae* is found in *Guidelines for the laboratory diagnosis of diphtheria*.[1]

Susceptibility testing

Routine susceptibility tests on throat or pharyngeal isolates are most often not required, and may even be misleading. The major pathogens involved in bacterial pharyngitis are *S. pyogenes* and *C. diphtheriae* benzylpenicillin and erythromycin are considered as the antimicrobials of choice to treat both types of infection. In cases of diphtheria, treatment with antitoxin is also indicated.

[1] Begg N. *Manual for the management and control of diphtheria in the European region*. Copenhagen, WHO Regional Office for Europe, 1994.

Lower respiratory tract infections

Introduction

Lower respiratory tract infections (LRTI) are infections occurring below the level of the larynx, i.e. in the trachea, the bronchi, or in the lung tissue (tracheitis, bronchitis, lung abscess, pneumonia). Sometimes, in pneumonia, the adjacent membranous covering of the lung is involved, resulting in roughening (pleurisy) and sometimes production of fluid in the pleural cavity (pleural effusion).

A special form of LRTI is pulmonary tuberculosis, which is common in many countries. The patient may cough up aerosols containing tubercle bacilli (*Mycobacterium tuberculosis*) which can be inhaled by other people. This form of the disease ("open" tuberculosis) is easily spread from person to person, and is therefore a serious communicable disease.

Many patients with LRTI cough up purulent (pus-containing) sputum that is generally green or yellowish in colour; this sputum may be cultured and examined grossly and microscopically.

There are other infections in which little or no sputum is produced: Legionnaire disease (caused by *Legionella pneumophila*), pneumonia due to *Mycoplasma pneumoniae* ("primary atypical pneumonia"), and *Chlamydia* pneumonia. These diseases require specialized techniques (serology and isolation on special cultures) for their diagnosis and will not be discussed further here. Apart from pulmonary tuberculosis (see below), most requests for sputum microscopy and culture concern patients with respiratory infections associated with purulent sputum.

The most common infections

Acute and chronic bronchitis

In patients with *acute bronchitis* (usually following an acute viral infection, such as a common cold or influenza), sputum is not usually cultured unless the patient fails to show signs of clinical improvement.

Chronic bronchitis is a long-lasting, disabling respiratory disease with periodic acute attacks. Most patients generally cough up sputum every day, which is usually grey and mucoid; the disease also has episodes when the condition of the patient becomes worse and obviously purulent sputum is coughed up. This is termed an acute exacerbation of chronic bronchitis. The typical respiratory pathogens (*Haemophilus influenzae*, *Streptococcus pneumoniae*, or less often *Moraxella* (*Branhamella*) *catarrhalis*) are frequently found in sputum samples.

Lung abscess

An abscess may form in the lung following the inhalation of a foreign body, of the stomach contents, or of upper respiratory tract (mouth or throat) secretions. This is sometimes termed "aspiration pneumonia". Attempts may be made to culture coughed-up sputum (which is often extremely foul-smelling),

but when there is an abscess (as demonstrated by radiography) the pus contained in it should be examined microscopically and cultured. Unfortunately, there is no medical agreement on how this pus should be obtained, but direct puncture and withdrawal of pus is one of the possibilities. Anaerobic bacteria such as *Prevotella melaninogenica* (formerly *Bacteroides melaninogenicus*) and *Peptostreptococcus* spp., derived from the mouth or throat flora, are often very important causative agents. Pus should be collected, transported, and examined according to standard methods for anaerobic culture of pus (see p. 86 and pp. 98–102).

Pneumonia and bronchopneumonia

Acute lobar pneumonia usually affects only a single lobe of the lung. This infection is nearly always caused by *S. pneumoniae*. This form of pneumonia occasionally occurs in epidemic form. A rare cause of a rather similar form of pneumonia is *Klebsiella pneumoniae*.

While a few patients infected with *S. pneumoniae* or *K. pneumoniae* will have classical pneumonia, the most frequent form of the disease is bronchopneumonia, with patches of infiltration and inflammation (termed "consolidation") distributed over one or often both lungs.

Many different kinds of viruses or bacteria can be associated with bronchopneumonia. Apart from *S. pneumoniae*, and sometimes *H. influenzae*, *Staphylococcus aureus* is a cause of bronchopneumonia, particularly during influenza or measles epidemics. Gram-negative rods (in particular, *E. coli* and *K. pneumoniae*) and *P. aeruginosa* are also frequently found. These infections are all common in intensive-care departments, especially when broad-spectrum antibiotics are widely used or mechanical respiration is carried out, and are indicative of indiscriminate use of antibiotics and failure to monitor patients carefully for early signs of infection.

If there is a pleural effusion, the fluid should be examined microscopically and cultured according to the procedures described for pus and exudates.

Pulmonary tuberculosis

The sputum of patients with pulmonary tuberculosis is usually not highly purulent, but should not be rejected for tuberculosis investigation because of this. An acid-fast stained smear (Ziehl–Neelsen) should be examined microscopically to detect immediately any patients who have acid-fast bacteria in their sputum[1]. After the smear has been stained, the sputum should be treated by a decontamination procedure (see p. 72) in order to kill as many of the non-mycobacterial organisms as possible and to leave the tubercle bacilli viable and thus suitable for culture on Löwenstein–Jensen medium.

Because the bacteriological procedures for the diagnosis of pyogenic respiratory infections, such as bronchitis and pneumonia, are so fundamentally different from those for tuberculosis, they will be considered separately. The physician must make it clear to the laboratory whether he or she wishes examinations for:

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[1] See *Manual of basic techniques for a health laboratory*, 2nd ed. Geneva, World Health Organization, 2003.

- pyogenic bacteria (*H. influenzae*, *S. pneumoniae*, etc.),
- tubercle bacteria (*M. tuberculosis*), or
- both types of bacteria.

Collection of sputum specimens

The collection of good sputum specimens is an art in itself and has been described in other books[1]. Examination of a badly collected sputum specimen can give misleading results because of contamination with the normal bacterial flora present in the mouth and throat; "sputum" consisting of saliva and food particles should not be examined.

The sputum should be collected in a sterile wide-mouthed container with a secure, tight-fitting cover and sent to the laboratory without delay. If the sputum is allowed to stand after collection, overgrowth of contaminating bacteria may take place before the examination is carried out and the results of smears and cultures will be highly misleading. For this reason, it is not recommended that sputum specimens be sent to the laboratory by mail. The only exceptions are specimens for tuberculosis examination that may have to be sent to a district or regional laboratory. The local and national postal regulations for the transmission of infected (pathological) material must be strictly applied.

Processing of sputum in the laboratory (for non-tuberculous infections)

After collection sputum must be immediately processed or kept in a refrigerator.

Macroscopic evaluation

The macroscopic appearance of the sputum should be recorded. Possible descriptions include:

purulent, green
purulent, yellow
mucopurulent (i.e. partially mucoid and partially purulent)
blood-stained
blood-stained, with green floccules
*grey, mucoid
*grey, frothy
*white, mucoid
*white, frothy
*white, mucoid, with some food particles
*watery (i.e. only saliva present)
*watery, with some food particles

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[1] *Specimen collection and transport for microbiological investigation*. Alexandria, WHO Regional Office for the Eastern Mediterranean, 1995 (WHO Regional Publications, Eastern Mediterranean Series 8).

Manual of basic techniques for a health laboratory, 2nd ed. Geneva, World Health Organization, 2003.

Technical guide for sputum examination for tuberculosis by direct microscopy. *Bulletin of the International Union Against Tuberculosis and Lung Disease*, 4th ed. 1996.

Sputum specimens marked with an asterisk should not normally be examined for non-tuberculous infections.

Microscopic examination

A portion of the purulent or mucopurulent sputum should be used for the preparation of a Gram-stained smear.

If no floccules of pus can be seen (e.g. in a grey mucoid sputum sample), the Gram-stained smear may show only the presence of large, rather square, squamous epithelial cells, frequently covered with masses of adherent bacteria. This is an indication that the specimen consists mainly of mouth or throat secretions, and culture should not be carried out as it is not relevant, and usually highly misleading. An accepted guideline is to reject, for culture, any specimen that contains fewer than 10 polymorphonuclear neutrophils per epithelial cell[1].

In many patients with acute respiratory infections (e.g. pneumonia) and purulent sputum, the emergency examination of a Gram-stained smear may provide guidance to the clinician in the choice of antimicrobial chemotherapy. Possible results include:

- Gram-positive diplococci surrounded by an empty space from the unstained capsules (suggestive of *S. pneumoniae*);
- small Gram-negative coccobacilli (probably *H. influenzae*);
- Gram-negative diplococci, intracellular and extracellular (suggestive of *Movaxella catarrhalis*;
- Gram-positive cocci in grape-like clusters (suggestive of *S. aureus*);
- Gram-negative rods (suggestive of the presence of Enterobacteriaceae or *Pseudomonas* spp.);
- large Gram-positive yeast-like cells, often with mycelia (suggestive of the presence of *Candida* spp.).

Cultural procedures and interpretation

When microscopy of the specimen demonstrates an acceptable quality of the sputum, select a floccule of purulent material (or of the most nearly purulent material available) using a sterile swap or loop and inoculate on to the various culture plates.

A suggested routine set of culture media is as follows:

- blood agar, with a streak of *S. aureus* to facilitate satellite growth of *H. influenzae*, and with an optochin disc placed in the middle of the secondary streaking,
- chocolate agar,
- MacConkey agar.

The blood agar and chocolate agar plates are incubated at 35–36 °C in an atmosphere containing extra carbon dioxide (e.g. in a candle jar) and the MacConkey plate is incubated in air.

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[1] Heinemann HS & Radano RR. Acceptability and cost savings of selective sputum microbiology in a community teaching hospital. *Journal of Clinical Microbiology*, 1979, **10**: 567–573.

If grape-like clusters of Gram-positive cocci were present in the stained smear, an extra mannitol salt agar (MSA) plate is suggested. The presence of Gram-positive, yeast-like structures in the stained smear may be an indication for the inoculation of a tube of Sabouraud dextrose agar (which needs to be incubated for at least 3 days at 35–37 °C). MSA and Sabouraud cultures do not need to be done routinely for all sputum specimens.

Cultures should be inspected after incubation overnight (18 hours) but reincubation for an extra 24 hours may be indicated when growth is less than expected from the microscopic findings, or when only tiny colonies are present.

Typical findings include the following:

- Flat, clear colonies with concave centres and zones of green (α-) haemolysis, as well as a zone of inhibition of growth around the optochin disc, may be *S. pneumoniae*. If the reading of the optochin test result on the primary plate is inconclusive, the test should be repeated on a subculture. It should not be forgotten that other α-haemolytic colonies (the so-called viridans streptococci) are normally present in the flora of the mouth and throat.
- Tiny, water-drop colonies growing as non-haemolytic satellite colonies on the blood agar plate, but much larger clear colonies on the chocolate agar or enriched blood agar plates, suggest the presence of *H. influenzae*. These colonies are usually present in large numbers, generally more than 20 per plate. Some laboratories choose to confirm this by X and V factor dependence tests, but these have to be very carefully controlled and are not strictly necessary. Serological typing of respiratory strains is usually not helpful, as most of them are "rough" and untypable.
- Brittle, dry, grey-white colonies on blood agar and chocolate agar plates that can be moved intact with a loop may indicate *M. catarrhalis*. If desired, a set of sugar degradation tests may be set up (all test results negative), but most laboratories do not do this. *Moraxella* organisms are strongly oxidase-positive, and their colonial and microscopic appearance is highly characteristic. As the morphologcal appearance of *Moraxella* resembles *Neisseria* spp. the tributyrin test may be used for differentiation, since *Moraxella* hydrolyses tributyrin.
- Medium-sized, golden-buff colonies are formed by *S. aureus*. The coagulase and the mannitol fermentation tests are positive, although the slide coagulase test ("bound" coagulase test) is occasionally negative. If there is a contradiction between the appearance of the colonies and the slide test, then a tube coagulase ("free" coagulase) test should be performed.
- Colonies on MacConkey agar suggest that Enterobacteriaceae or *Pseudomonas* spp. or *Acinetobacter* spp. are present.
- Whitish, round, matt colonies on the blood agar and chocolate agar plates may be *Candida albicans*, which will also grow within 2–3 days on a Sabouraud dextrose agar culture.

It should be stressed that rare colonies of any of the above organisms either stem from the normal commensal flora of the respiratory tract, or are a result of colonization (e.g. coliforms, yeasts). As they may not be relevant to the management of the patient, they should not be reported, or should be reported as colonizing flora.

Susceptibility testing

Susceptibility tests should be performed only when the amount of growth is considered significant, and not on every bacterial species present in small

Table 20. Interpretation of susceptibility test results of fastidious organisms[a]

	Total zone diameter (mm)		
	Resistant	**Intermediate**	**Susceptible**
S. pneumoniae (Mueller–Hinton with 5% sheep blood in 5% CO_2 incubation			
Oxacillin (1 μg) (for benzylpenicillin)	⩽19[b]	—	⩾20
Tetracycline (30 μg)	⩽18	19–22	⩾22
Erythromycin (15 μg)	⩽15	16–20	⩾21
Chloramphenicol (30 μg)	⩽20	—	⩾21
Co-trimoxazole (25 μg)	⩽15	16–18	⩾19
M. catarrhalis (Mueller–Hinton)			
Tetracycline (30 μg)	⩽14	15–18	⩾19
Erythromycin (15 μg)	⩽13	14–22	⩾23
Co-trimoxazole (25 μg)	⩽10	11–15	⩾16

[a]National Committee for Clinical Laboratory Standards (NCCLS). *Performance standards for antimicrobial susceptibility testing*. M100-S8. Vol 18, 1998.
[b]Resistant or intermediate.

numbers in the culture. Interpretations of some possible results are presented in Table 20.

For Enterobacteriaceae and staphylococci the standardized disc-diffusion method (Kirby–Bauer) should be used. Strains of *S. pneumoniae* should be tested on Mueller–Hinton agar, supplemented with 5% sheep blood, for susceptibility to tetracycline, chloramphenicol, erythromycin, and benzylpenicillin. Conventional blood agar can also be used. For benzylpenicillin, a disc containing 1 μg of oxacillin is preferred to a disc containing benzylpenicillin itself as results with this oxacillin agree better with the MIC value for benzylpenicillin; it is also more stable. Benzylpenicillin discs may deteriorate rapidly in hot climates and thus produce unreliable results.

H. influenzae strains should be tested for β-lactamase production, using, for example, the Nitrocefin test. Rare strains of *H. influenzae* may be ampicillin-resistant without producing β-lactamase. At this stage it is not recommended to test *H. influenzae* for antibiotic susceptibility by the disc-diffusion technique.

M. catarrhalis isolates should be tested for β-lactamase production. Testing against tetracycline and erythromycin is optional.

Candida albicans cultures need not be tested against any antimicrobial agents.

Most laboratories give a semi-quantitative assessment of the bacteria cultured on solid media, which might be presented as follows:

(+) = few colonies
+ = light growth
+ + = moderately heavy growth
+ + + = heavy growth.

Culture for Mycobacterium tuberculosis

In addition to the preparation of a direct, acid-fast stained smear, material (usually, but not always, sputum) should be cultured for *M. tuberculosis*, whenever this disease is clinically suspected. Some patients, in whom pulmonary tuberculosis is suspected, may not cough up any sputum. A little sputum may, in fact, be produced but is immediately swallowed. In this case, the physician should collect a specimen of fasting gastric juice (generally obtained early in the morning) and neutralize the material using sodium bicarbonate (100 mg) before sending it to the laboratory. The gastric juice should be treated in the same way as sputum. It is expensive to culture all sputum samples routinely for tubercle bacilli (although some unsuspected patients would be discovered); therefore this is not routinely recommended.

Concentration–digestion–decontamination procedures

Sputum from patients with tuberculosis infection often contains solid particles of material from the lungs and this material should be selected for culture, whenever it is found. However, as tuberculous sputum is coughed up through the throat and mouth, contamination with the normal flora of the pharynx is inevitable. The contaminating bacteria must be killed if the Löwenstein–Jensen culture media are not to become overgrown. A concentration-digestion-decontamination procedure of any specimen collected from a site where there are normal flora is therefore recommended. The following three procedures are widely used:

— Sodium hydroxide (NaOH) (Petroff);
— *N*-acetyl-L-cysteine–sodium hydroxide (NALC–NaOH); and
— Zephiran–trisodium phosphate

Sodium hydroxide procedure (Petroff)

This procedure liquifies the sometimes mucoid sputum while destroying the contaminating organisms. However, sodium hydroxide is also toxic for mycobacteria, and care must be taken when using the method to ensure that:

— the final concentration of NaOH does not exceed 2%;
— the tubercle bacilli are not exposed to sodium hydroxide for more than 30 minutes, including centrifugation time.

1. Mix equal volumes of sputum and 4% sodium hydroxide 40 g/l (previously sterilized by autoclaving) in a sterile, leak-proof, 50-ml glass bottle or jar, or plastic conical centrifuge tube.
2. Incubate at room temperature (25–30 °C) for 15 minutes, shaking the mixture carefully every 5 minutes using a mechanical shaker. In hot climates some cooling may be needed or the reaction time may be reduced to 10–15 minutes.
3. Centrifuge immediately or dilute the mixture to the 50-ml mark with distilled water or phosphate buffer (pH 6.8) to stop the action of the NaOH.
4. After 15 minutes, centrifuge the specimen at 3000 *g* for 15 minutes. Discard the supernatant carefully into a splash-proof container filled with a suitable disinfectant (phenol- or glutaraldehyde-based). Neutralize the sediment drop by drop with a 2-mol/l HCl solution containing 2% of phenol red, combined with shaking, until the colour changes persistently from red to yellow. Alternatively add one drop of indicator solution and then add HCl drop by drop while shaking continuously.

5. If the media is to be inoculated immediately, suspend the neutralized deposit in 1–2 ml of sterile 0.85% NaCl or sterile distilled water. Otherwise, suspend the sediment in 1–2 ml of sterile bovine albumin fraction V.

N-acetyl-L-cysteine–sodium hydroxide procedure

A lower concentration of NaOH in the presence of a mucolytic agent like *N*-acetyl-L-cysteine (NALC) is less aggressive against tubercle bacilli. Neither the incubation time nor the temperature are as crucial as in the NaOH procedure. However, the short shelf-life of no more than 24 hours of the NALC–NaOH working solution requires daily preparation.

1. Combine equal volumes of sodium citrate solution (29 g sodium citrate dihydrate per litre of distilled water) and 4% sodium hydroxide (40 g/l), and autoclave the mixture. The solution may be stored at room temperature.
2. Just prior to use, add 0.5 g NALC to 100 ml of NaOH–sodium citrate solution.
3. Depending on the number of specimens that must be decontaminated, prepare 2.5 g of NALC in 500 ml of NaOH–sodium citrate solution or 5 g NALC in 1000 ml of NaOH–sodium citrate solution. After 24 hours the reagent must be discarded.
4. Add an equal volume of NALC–NaOH working solution to the specimen in a sterile, leak-proof, 50-ml glass bottle or jar, or plastic conical centrifuge tube. Securely tighten the screw-cap, invert the tube and shake it gently for no longer than 30 seconds.
5. Let the tube stand for 15 minutes at room temperature (20–25 °C).
6. Dilute the mixture to the 50-ml mark with distilled water or with 67 mmol/l phosphate buffer (pH 6.8) to stop the action of the NaOH. Discard the supernatant carefully into a splash-proof container filled with a suitable disinfectant (phenol- or glutaraldehyde-based).
7. If the media is to be inoculated immediately, suspend the deposit in 1–2 ml of sterile 0.85% NaCl or sterile distilled water. Otherwise, suspend the sediment in 1–2 ml of sterile bovine albumin fraction V.

Zephiran–trisodium phosphate procedure

Mycobacteria can withstand prolonged treatment with this more gentle procedure. Therefore, incubation time and temperature are not critical.

1. Prepare 1 kg of trisodium phosphate ($Na_3PO_4 \cdot 12H_2O$) in 4 litres of hot distilled water, add 7.5 ml of 17% benzalkonium chloride (Zephiran), mix well, and store at room temperature.
2. Mix an equal volume of sputum (up to 10 ml) with the Zephiran–trisodium phosphate solution in a 50-ml sterile, leak-proof centrifugation glass bottle. Tighten the cap and vigorously shake the mixture manually or using a mechanical shaker for 30 minutes.
3. Leave the mixture to stand for an additional 30 minutes.
4. Centrifuge at 3000 *g* for 15 minutes. Discard the supernatant carefully into a splash-proof container filled with a suitable disinfectant (phenol- or glutaraldehyde-based), and re-suspend the sediment in 20 ml of neutralizing phosphate buffer, pH 6.6[1].

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[1] Preparation of neutralizing phosphate buffer 67 mmol/l.
Stock solutions:
A. Dissolve 9.47 g of anhydrous disodium phosphate in 1 litre of distilled water.
B. Dissolve 9.07 g of anhydrous monopotassium phosphate in 1 litre of distilled water.
For pH 6.8 buffers: mix 50 ml of stock solution A with 50 ml of stock solution B.
For pH 6.6 buffers. mix 37.5 ml of stock solution A with 62.5 ml of stock solution B.
Check the pH. Add solution A to raise the pH, or solution B to lower the pH as necessary.

5. Centrifuge once again at 3000*g* for 15 minutes. Discard the supernatant and inoculate the sediment onto the media.

Culture

1. Inoculate 3 drops (about 0.1 ml) of the sediment onto at least three plates of Löwenstein–Jensen medium or equivalent.
2. Determine the contamination rate of the incubated media regularly and record the number of contaminated plates.

The rate of contamination should be 3–5%. Excessive contamination (over 5%) of the Löwenstein–Jensen cultures usually indicates that the decontamination procedure was not effective enough. Contamination rates of <3% suggest that the decontamination procedure was too vigorous and mycobacteria present in the samples may fail to grow.

Interpretation of cultures for M. tuberculosis

The tubes containing the Löwenstein–Jensen medium should be incubated for 2–3 days at 35–37 °C in a horizontal position, with the tops loosened half a turn. The culture tubes should then be stored at 37 °C for six weeks and inspected for growth at weekly intervals. During these weekly inspections, the growth of any colonies of bacteria on the surface should be noted. A smear should be carefully made and stained by the Ziehl–Neelsen procedure. If the organisms are not acid-fast bacilli, then the culture may be recorded as contaminated.

Typical human strains of *Mycobacterium tuberculosis* are "rough, tough and buff", and can sometimes be seen after 2–3 weeks of incubation (but seldom earlier). Bovine strains (*M. bovis*) are generally smooth and whitish-cream in colour. Other, generally nonpathogenic, mycobacterial species may grow more quickly (sometimes after only several days) and may or may not produce pigmented growth (red, yellow, or orange). If an isolate has the typical colonial appearance and the Ziehl–Neelsen stained smear from a colony is also typical, it should be reported as "*Mycobacterium* spp., probably *M. tuberculosis*"; the isolate should also be sent to the national or local reference laboratory for identification and susceptibility testing, as these are specialized procedures.

General note on safety

Sputum should always be treated with care, and leak-proof specimen containers should be used. This is particularly important if the postal service has to be used. It is advisable that *all* procedures involving sputum (even when tuberculosis is not mentioned on the request form) are carried out in a bacteriological safety box. Even a home-made version is better than none at all. It is strongly recommended that laboratories that process specimens supposedly containing pathogenic mycobacteria meet at least the biosafety level 2 requirements.[1]

Particular care must be taken when opening, closing, or shaking bottles, and when centrifuging materials. The production of infected aerosols may infect

[1] *Manual of basic techniques for a health laboratory*, 2nd ed. Geneva, World Health Organization, 2003.

laboratory personnel and appropriate occupational health procedures should be applied.[1]

Transportation of cultures of *M. tuberculosis* by mail to the national reference laboratory presents special risks in the event of accidents or breakage of the container. Only approved containers and dispatch materials conforming to postal requirements should be used.

[1] *Laboratory services in tuberculosis control Part I: Organization and management*. Geneva, World Health Organization, 1998 (unpublished document WHO/TB/98.258).

Sexually transmitted diseases

Introduction

The number of microorganisms known to be sexually transmitted or transmissible, and the spectrum of clinical syndromes associated with these agents, have expanded enormously during the past twenty years. Table 21 lists selected sexually transmissible microorganisms and the diseases they cause. The etiological diagnosis of some of these conditions is a major challenge to the clinical microbiology laboratory. A laboratory diagnosis is an essential component in the management and control of diseases such as gonorrhoea and syphilis, and has implications not only for the patient but also for his or her sex partners.

This section discusses briefly the identification of the most commonly occurring sexually transmissible microorganisms found in specimens from the female and male genital tract. Viruses and bacterial agents such as *Ureaplasma urealyticum*, *Mycoplasma hominis*, and *Mobiluncus* spp. will not be dealt with

Table 21. Selected sexually transmissible microorganisms and related syndromes

Etiological agent	Syndrome
Neisseria gonorrhoeae	Bartholinitis, cervicitis, chorio-amnionitis, conjunctivitis, disseminated gonococcal infection (arthritis, dermatitis, tenosynovitis), endometritis, epididymitis, infertility, pharyngitis, prepubertal vaginitis, perihepatitis, proctitis, prostatitis, salpingitis, urethritis
Chlamydia trachomatis (serovars D–K)	Bartholinitis, cervicitis, conjunctivitis in infants, endometritis, epididymitis, infant pneumonia, infertility, otitis media in infants, pelvic inflammatory disease (PID), perihepatitis, prepubertal vaginitis, proctitis, Reiter syndrome, salpingitis, urethritis
Chlamydia trachomatis (serovars L_1, L_2, L_3,)	Lymphogranuloma venereum
Treponema pallidum	Syphilis
Haemophilus ducreyi	Chancroid
Calymmatobacterium granulomatis	Granuloma inguinale (donovanosis)
Mobiluncus spp.	Bacterial vaginosis
Human (alpha) herpesvirus (HSV I and HSV II)	Genital and orolabial herpes, meningitis, neonatal herpes, proctitis
Cytomegalovirus (CMV)	Congenital infection
Human papilloma virus (HPV)	Cervical cancer, viral warts
Human immunodeficiency virus (HIV)	Acquired immunodeficiency syndrome (AIDS) and AIDS-related complex
Hepatitis B virus (HBV)	Hepatitis B
Gardnerella vaginalis	Urethritis, vaginitis
Candida albicans	Balanoposthitis, vulvovaginitis

here. For more extensive information, the reader is referred to the relevant WHO publication.[1]

Urethritis in men

Urethritis in men is clinically characterized by a urethral discharge and/or dysuria, but asymptomatic infection with *Neisseria gonorrhoeae* or *Chlamydia trachomatis* occurs frequently. If untreated, gonococcal and chlamydial urethritis may progress to epididymitis. Rectal and oropharyngeal infection with *N. gonorrhoeae* and *C. trachomatis* may occur in homosexual men.

For the purpose of patient management, urethritis should be divided into gonococcal urethritis and nongonococcal urethritis (NGU). Approximately half the cases of NGU are caused by *C. trachomatis*, but the etiology of the majority of the remaining cases has not been fully elucidated. According to some studies, *Ureaplasma urealyticum* may be a cause of urethritis, and *Trichomonas vaginalis* can be found in 1–3% of cases of NGU. Intra-urethral infection with human herpesvirus may yield a urethral discharge. Bacterial agents such as staphylococci, various Enterobacteriaceae, *Acinetobacter* spp., and *Pseudomonas* spp. can be isolated from the urethra of healthy men, but have not been shown to cause urethritis.

The examination of specimens for *C. trachomatis* is complicated and will not be discussed in this section. Besides isolation in cell culture systems, non-culture methods for the detection of chlamydial antigens by enzyme immunoassays, immunofluorescence assays and nucleic acid amplification tests have recently become available. These methods, while promising, remain prohibitively expensive.

Collection and transport of specimens

For the collection of urethral specimens, a swab with a narrow diameter or a sterile bacteriological loop should be inserted 3–4 cm into the urethra and gently rotated before withdrawal. Purulent discharge can be collected directly on a swab or on the inoculating loop. The composition of both the tip and the shaft of the swab is important. For the culture of *N. gonorrhoeae*, charcoal-treated cotton tips or calcium alginate or Dacron tips are preferred. If these special, commercially prepared, sampling swabs are not available and regular cotton swabs are used, the specimen should be inoculated immediately. A prostatic massage does not increase the rate of isolation of gonococci or chlamydiae in cases of urethritis.

Anorectal specimens are obtained by inserting a swab 4–5 cm into the anal canal. For oropharyngeal specimens, the posterior pharynx and the tonsillar crypts should be swabbed and plated immediately.

Ideally the inoculation of specimens for the isolation of *N. gonorrhoeae* should be made directly onto the culture medium in the clinic. Inoculated plates should be placed in a candle jar or into an atmosphere containing 5–10% carbon dioxide, with high humidity. If immediate plating and incubation are not possible, a transport medium such as Amies or Stuart transport medium should be used. The transport time should be as short as possible, and must

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[1] Van Dyck E, Meheus AZ, Piot P. *Laboratory diagnosis of sexually transmitted diseases*. Geneva, World Health Organization, 1999.

be less than 12 hours in ambient temperatures up to 30 °C. Refrigeration is to be avoided.

Direct examination and interpretation

Most studies have shown that the presence of four or more polymorphonuclear (PMN) leukocytes per oil-immersion field is strongly indicative of urethritis in men. This criterion is particularly useful to the clinician who has to decide whether to treat patients with vague urethral complaints.

In most cases of gonorrhoea in the male, the discharge is purulent, and numerous polymorphonuclear leukocytes (>10 per oil-immersion field) can be seen in the urethral smear. However, this is not always the case in NGU, which yields a less severe inflammatory reaction. Smears with more than 4 PMN leukocytes per oil-immersion field, and without intracellular Gram-negative diplococci, are highly suggestive of NGU.

A thinly spread smear, prepared by rolling a swab over a slide, should be heat-fixed and methylene-blue or Gram-stained. The presence of Gram-negative intracellular diplococci in PMN leukocytes in a urethral smear is strongly suggestive of gonorrhoea.

Gram-stained smears of intra-urethral specimens from asymptomatic males, from blind rectal swabs, or from oropharyngeal samples are not recommended. However, microscopic examination of purulent material obtained under anoscopy has a fairly high diagnostic value.

Culture of *Neisseria gonorrhoeae*

Inoculated plates with modified Thayer–Martin (MTM)[1] agar (or New York City medium (NYC))[2] should be incubated at 35 °C in a humid atmosphere enriched with carbon dioxide (candle jar), and should be observed daily for two days. Laboratories processing a large number of specimens for *N. gonorrhoeae* often prefer to use a non-selective chocolate agar enriched with IsoVitaleX, or an equivalent supplement, in addition to the selective MTM, because as many as 3–10% of gonococcal strains in a given area may be susceptible to the concentration of vancomycin used in selective media.

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[1] Modified Thayer–Martin agar is prepared by adding at 50 °C a mixture of antimicrobials and IsoVitaleX, or an equivalent supplement, to chocolate agar prepared from GC agar or Columbia agar as basal medium. Antimicrobial mixtures containing 3 or 4 antimicrobials are commercially available from several sources; VCN mixture contains vancomycin, colistin, and nystatin; VCNT mixture also contains trimethoprim.
The final concentrations of the antimicrobials in the prepared medium are:

- vancomycin: 3 µg/ml
- colistin: 7.5 µg/ml
- nystatin: 12.5 IU/ml
- trimethoprim lactate: 5 µg/ml

[2] Modified New York City medium is prepared by adding 500 ml of sterile GC agar base cooled to 50 °C, the following supplements:

- 50 ml horse blood lysed by adding 5 ml/l saponin,
- sterile yeast autolysate,
- an antimicrobial mixture containing vancomycin, colistin, amphotericin, and trimethoprim.

The ingredients are commercially available from Oxoid Ltd, Wade Rd, Basingstoke, Hants RG24 8PW, England.

Gonococcal colonies may still not be seen after 24 hours. They appear after 48 hours as grey to white, opaque, raised, and glistening colonies of different sizes and morphology.

Identification of *Neisseria gonorrhoeae*

A presumptive identification of *N. gonorrhoeae* isolated from urogenital specimens is based on a positive oxidase reaction and a Gram-stained smear showing Gram-negative diplococci. Confirmation of the identification can be obtained by carbohydrate degradation assays or other tests, using methods and media discussed extensively elsewhere.[1]

Antimicrobial susceptibility testing

There is considerable geographical variation in the susceptibility of *N. gonorrhoeae* strains to benzylpenicillin. In some areas, such as sub-Saharan Africa or South-east Asia, most gonococcal strains are now β-lactamase-producing. Chromosomally mediated resistance to benzylpenicillin not based on β-lactamase production is also becoming more common in many countries. However, the disc-diffusion test is not reliable in detecting such strains.

In areas where benzylpenicillin, ampicillin or amoxicillin is still used for the treatment of gonococcal infections, *N. gonorrhoeae* isolates (particularly from cases of treatment failure) should be routinely screened for β-lactamase production by one of the recommended tests, such as the Nitrocefin test.[2] For the Nitrocefin test, a dense suspension from several colonies is prepared in a small tube with 0.2 ml saline; 0.025 ml of Nitrocefin is then added to the suspension and mixed for one minute. A rapid change in the colour, from yellow to pink or red, indicates that the strain produces β-lactamase.

Antimicrobial susceptibility testing of *N. gonorrhoeae* by the disc-diffusion assay is not recommended in routine practice.

Genital specimens from women

The vaginal flora of premenopausal women normally consists predominantly of lactobacilli, and of a wide variety of facultative aerobic and anaerobic bacteria.

Abnormal vaginal discharge may be due to:

— vaginitis: *Gardnerella vaginalis, Candida albicans*;
— bacterial vaginosis: overgrowth of anaerobes and *Mobiluncus* spp.;
— cervicitis: *Neisseria gonorrhoeae, Chlamydia trachomatis*.

Other bacteria, such as Enterobacteriaceae, are not proven causes of vaginitis. Vaginitis in prepubertal girls may be due to *N. gonorrhoeae* or *C. trachomatis*.

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[1] Van Dyck E, Meheus AZ, Piot P. *Laboratory diagnosis of sexually transmitted diseases*. Geneva, World Health Organization, 1999.

[2] The Nitrocefin reagent is obtainable from Oxoid Ltd, Wade Road, Basingstoke, Hants RG24 8PW, England, and consists of 1 mg of Nitrocefin (SR112) and 1 vial of rehydration fluid (SR112A). The tube test can be replaced by a disc test, using Nitrocefin-impregnated paper discs (Cefinase discs, available from BD Diagnostic Systems, 7 Loveton Circle, Sparks, MD 21152, USA).

Bacterial vaginosis (nonspecific vaginitis) is a condition characterized by an excessive, malodorous, vaginal discharge associated with a significant increase of *Mobiluncus* spp. and various obligate anaerobes, and a decrease in the number of vaginal lactobacilli. A minimum diagnostic requirement for bacterial vaginosis is the presence of at least three of the following signs: abnormal vaginal discharge, vaginal pH > 4.5, clue cells (epithelial cells with so many bacteria attached that the cell border becomes obscured), and a fishy, amine-like odour when a drop of 10% potassium hydroxide is added to the vaginal secretions.

Urethritis in women is also often caused by *N. gonorrhoeae* and *C. trachomatis*.

Ascending infections with *N. gonorrhoeae, C. trachomatis*, vaginal anaerobes, and facultative anaerobic bacteria can cause pelvic inflammatory disease (PID), with infertility or ectopic pregnancy as late sequelae.

Genital infections with bacterial agents, including *N. gonorrhoeae* and *C. trachomatis*, during pregnancy may result in complications such as premature delivery, prolonged rupture of membranes, chorio-amnionitis, and postpartum endometritis in the mother, and conjunctivitis, pneumonia, and amniotic infection syndrome in the newborn.

On special request, cervicovaginal specimens may be cultured for bacterial species, such as *S. aureus* (toxic shock syndrome), *S. agalactiae* (group B streptococci, neonatal infection), *Listeria monocytogenes* (neonatal infection), and *Clostridium* spp. (septic abortion).

Although infections with *C. trachomatis* and with human herpesvirus are common, and can cause significant morbidity, their laboratory diagnosis requires expensive equipment and reagents and will not be discussed here.

Collection and transport of specimens

All specimens should be collected during a pelvic examination using a speculum. The speculum may be moistened with warm water before use, but antiseptics or gynaecological exploration cream should not be used, since these may be lethal to gonococci.

For examination for yeasts, *G. vaginalis*, and bacterial vaginosis, samples of vaginal discharge may be obtained with a swab from the posterior fornix of the vagina. Samples for gonococcal and chlamydial culture should be collected in the endocervix. After inserting the speculum, cervical mucus should be wiped off with a cotton wool ball. A sampling swab (see page 77) should then be introduced into the cervical canal and rotated for at least 10 seconds before withdrawal.

Urethral, anorectal, and oropharyngeal specimens for gonococci may be obtained in a similar manner as from males.

In all cases of pelvic inflammatory disease (PID), as a minimum, the cervix should be sampled for *N. gonorrhoeae*. Sampling from the fallopian tubes is more reliable, but in most areas a cul-de-sac aspirate is the best sample available.

In infants with ophthalmia neonatorum, conjunctival exudate should be collected with a swab or a loop.

Amies and Stuart transport media are convenient for transport of cervical and vaginal samples, with the exception of specimens to be tested for *C. trachomatis*.

Direct examination and interpretation

Direct examination of vaginal secretions is the method of choice for the etiological diagnosis of vaginitis, but is much less useful for the diagnosis of cervicitis.

A wet mount is prepared by mixing the vaginal sample with saline on a glass slide, after which a cover slip is added. A diluted preparation is preferred to ensure the separation of the cells, which may otherwise be clumped together. Examine at a magnification of ×400 for the presence of *T. vaginalis* with typical movement, budding yeasts, and clue cells. *C. albicans* may form pseudomycelia, which may be observed occasionally in vaginal material. Clue cells are found in most women with bacterial vaginosis. A granular or dirty appearance of the epithelial cell cytoplasm is a less objective criterion than the loss of the cell border. Microscopic examination of a wet mount of cervical specimens is not recommended.

Preparation of a Gram-stained smear is the method of choice for the diagnosis of bacterial vaginosis. The smear should be prepared by gently rolling, rather than smearing, a swab over the glass slide. A normal vaginal smear contains predominantly lactobacilli (large Gram-positive rods) and fewer than 5 leukocytes per field. In typical smears from women with bacterial vaginosis, clue cells covered with small Gram-negative rods are accompanied by a mixed flora consisting of very large numbers of small Gram-negative and Gram-variable rods and coccobacilli, and often Gram-negative curved rods, in the absence of larger Gram-positive rods. Only a few (<5) leukocytes are found per field. This picture is a sensitive and specific diagnostic indicator for bacterial vaginosis.

A large number of white blood cells (>10 cells per field) on the Gram-stained vaginal smear suggests trichomoniasis or cervicitis.

Gram-staining is not particularly helpful for the diagnosis of gonococcal infection in female patients. The examination of Gram-stained smears of endocervical secretions for intracellular Gram-negative diplococci has a sensitivity of 50–70%, and a specificity of 50–90% for the diagnosis of gonococcal infection, resulting in a poor predictive value of a positive test in populations with a low prevalence of gonorrhoea. Gram-negative intracellular diplococci in cervical smears should be reported as such, and not as *N. gonorrhoeae* or gonococci. Over-interpretation of cervical smears, which often contain Gram-negative coccobacilli and bipolar stained rods, must be avoided.

The main interest in a cervical smear is its validity for the diagnosis of mucopurulent cervicitis: the presence of more than 10 polymorphonuclear leukocytes per oil-immersion field is a reasonably good indication of mucopurulent cervicitis, most often due to *N. gonorrhoeae* and/or *C. trachomatis*.

The examination of a Gram-stained conjunctival smear is a sensitive and specific technique for the diagnosis of gonococcal conjunctivitis. The presence of intracellular Gram-negative diplococci is diagnostic for gonococcal conjunctivitis.

Culture

Cervical, rectal, urethral, conjunctival, and cul-de-sac specimens may be cultured for *N. gonorrhoeae* using the methods specified on page 78. Specimens should be processed as soon as they arrive in the laboratory or, preferably, in the clinic itself. Unlike in males, culture is essential for the diagnosis of gonococcal infection in females. The sensitivity of a single culture for the diagnosis of gonorrhoea in women is 80–90%. The sensitivity is lower for specimens taken during the peripartum period.

Cultures for *G. vaginalis* or anaerobes are not recommended for the diagnosis of bacterial vaginosis, since the organisms are recovered from 20–40% of women without vaginal infection. The presence of *G. vaginalis* in vaginal discharge is in itself not an indication for treatment, and only patients fulfilling the diagnostic criteria for bacterial vaginosis should be treated for this condition.

As compared with microscopy, cultures increase the detection of *C. albicans* by 50–100%. Culture methods are usually more efficient when the number of organisms is low. However, low numbers of *C. albicans* can be found in the vagina of 10–30% of women without signs or symptoms of vaginitis, and only large numbers of *C. albicans* should be considered as evidence of vaginal candidiasis. Consequently, culture is not recommended. Cultures for *G. vaginalis* will mainly detect asymptomatic carriers when performed in addition to a wet mount, and should not be performed.

Specimens from genital ulcers

Genital ulcers are a very common problem in many developing countries. Their etiological diagnosis and management are a challenge to the clinician as well as to the laboratory. Mixed infections are common. Genital ulcerative lesions can be caused by a variety of sexually transmissible agents:

— human herpesvirus
— *Treponema pallidum*
— *Haemophilus ducreyi*
— *Calymmatobacterium granulomatis*, the agent of granuloma inguinale (Donovanosis)
— *Chlamydia trachomatis* serovars L_1, L_2, L_3

Genital herpes is the most common cause of genital ulcer disease in most industrialized countries, and is a cause of life-threatening complications in immunodeficient patients and neonates born to women with the infection. Its laboratory diagnosis will not be discussed here.

Syphilis is still the most serious disease associated with genital lesions, since it can give rise to severe late sequelae and to congenital syphilis. While serological tests play an important role in the diagnosis of all stages of syphilis, only the dark-field examination will be discussed here. Techniques and interpretation of serological tests for syphilis have been extensively reviewed elsewhere.[1]

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[1] Van Dyck E, Meheus AZ, Piot P. *Laboratory diagnosis of sexually transmitted diseases*. Geneva, World Health Organization, 1999.

Chancroid (ulcus molle) is the major cause of genital ulceration in many developing areas. The clinical features include painful, purulent ulcer(s) accompanied by painful and occasionally suppurative inguinal buboes. Late sequelae are not known to occur. The clinical differentiation from other genital ulcer diseases is difficult. Chancroid increases the risk of acquiring HIV infection.

Granuloma inguinale is characterized by extensive beefy, red, granulated genital ulcers. Bubo formation is rare.

Chlamydial lymphogranuloma is typically associated with inguinal and/or femoral lymphadenopathy, and, less frequently, with small ulcers which heal spontaneously. Its diagnosis is based on serological tests and isolation of *C. trachomatis* serovars L_1, L_2, L_3.

Collection of specimens

Treponema pallidum: Protective surgical gloves should be worn. Squeeze the ulcer between two fingers and clean the surface of the lesion with saline, using gauze swabs. Crusts should be removed if present. After wiping away the first drops of blood (if present), collect a sample of serous exudate by touching a completely clean glass slide to the surface of the lesion. Immediately place a clean coverslip firmly on the drop of exudate. Alternatively, the specimen may be aspirated from the lesion, or from an enlarged lymph node, using a sterile needle and syringe. The preparation should be examined immediately by a microscopist experienced in dark-field microscopy.

Haemophilus ducreyi: Specimens should be obtained from the base of the ulcer with a swab and inoculated directly on to the isolation medium. Material may also be aspirated from inguinal buboes, but isolation of *H. ducreyi* is less successful than from genital lesions. Transport media for *H. ducreyi* have not been evaluated.

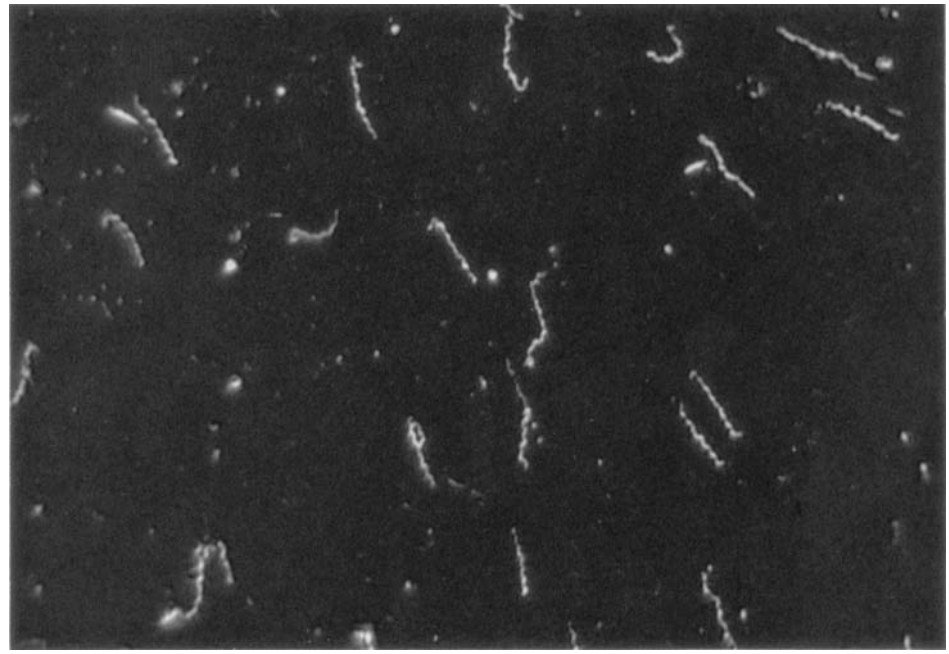
If granuloma inguinale is suspected, ideally a biopsy of subsurface tissue from an area of active granulation should be made. Fresh smears should be made from a crushed piece of biopsy material. Alternatively, one may make a smear by scraping off the surface of the lesion.

Direct examination

Demonstration of treponemas in lesion material is the method of choice for the diagnosis of primary syphilis. Although *T. pallidum* can be stained (for instance with silver nitrate), dark-field microscopy is recommended because it is more sensitive and specific. A microscope equipped with a good light source and a dark-field condenser must be available for dark-field examination. Dark-field condensers block out the direct light rays, allowing only the peripheral rays (deflected by objects such as treponemes) to pass through.

Place a few drops of immersion oil on the condenser of a dark-field microscope. Lower the condenser slightly so that the oil is below the level of the stage. Place the slide on the microscope and raise the condenser until there is good contact between the oil and the underside of the slide. Carefully avoid trapping air bubbles in the oil.

Fig. 9. Appearance of *T. pallidum* under dark-field microscopy [Negative]



Use the low-power objective (×10) to bring the specimen into focus. Centre the light in the field by adjusting the centring screws located on the condenser, and focus the condenser by raising or lowering it until the smallest diameter of light is obtained. Recentre the light if necessary. Then use the dry ×40 objective to bring the specimen into focus, and examine the slide carefully. The contrast will be better when the microscopy is done in the dark. Avoid bright daylight.

T. pallidum appears white, illuminated on a dark background (Fig. 9). It is identified by its typical morphology, size, and movement. It is a thin (0.25–0.3 µm) organism, 6–16 µm long, with 8–14 regular, tightly wound deep spirals. It exhibits quick and rather abrupt movements. It rotates relatively slowly about the longitudinal axis (like a corkscrew). This rotation is accompanied by bending (twisting) in the middle and is executed rather stiffly. Lengthening and shortening (like an elastic expander spiral) may be observed. Distortion may occur in tortuous convolutions. When the organism is attached to, or obstructed by, heavier objects, the resulting vigorous struggling distorts the coils. Other non-syphilis spirochaetes may be loosely coiled, thick, and coarse; the movements are different (not like a corkscrew), but take the form of a more writhing motion, with marked flexion and frequent relaxation of the coils.

The demonstration of treponemes with morphology and motility characteristic of *T. pallidum* constitutes a positive diagnosis for primary and secondary syphilis. Patients with a primary chancre, which is dark-field positive, may be serologically negative. They normally become serologically reactive within a few weeks.

Failure to find the organism does not exclude a diagnosis of syphilis. Negative results may mean that:

- An insufficient number of organisms was present (a single dark-field examination has a sensitivity of no more than 50%).
- The patient had already taken antimicrobials.

- The lesion was approaching natural resolution.
- The lesion was not syphilitic.

Whatever the result of the dark-field examination, a blood sample should always be taken for serological tests.

In the diagnosis of granuloma inguinale, when acetone-fixed smears are stained with Giemsa, typical, intracellular, encapsulated bacilli can be seen within histiocytes. The diagnosis of this disease is described in full elsewhere.[1]

For the diagnosis of chancroid, a Gram-stained smear is not recommended, since the sensitivity and specificity are both less than 50%. For the diagnosis of chlamydial lymphogranuloma, Giemsa-stained smears are not recommended. However, material from the ulcer should also be examined by dark-field microscopy to search for *T. pallidum.*

Culture

Gonococci are occasionally isolated from genital ulcers, but their significance in such specimens is unclear. Apart from *H. ducreyi*, no other bacterial species—neither facultative aerobes nor obligate anaerobes—have been shown to cause genital ulcer disease.

Specimens to be examined for *H. ducreyi* should be inoculated directly on to a selective, enriched agar plate.[2] The medium used should not be older than one week. The plates should be incubated at 33–35 °C in a candle jar with a moistened towel at the bottom. After 48–72 hours of incubation, small, non-mucoid, yellow-grey, semi-opaque or translucent colonies appear that can be pushed intact across the agar surface. The sensitivity of a single culture for the isolation of *H. ducreyi* is 70–80%.

A presumptive diagnosis of *H. ducreyi* can be made on the basis of the characteristic morphology of colonies on selective media, and the demonstration of small Gram-negative, pleomorphic coccobacilli, often in single chains (streptobacilli), parallel chains ("school of fish"), or clumps in the suspect colonies. Although *H. ducreyi* is haemin-dependent, most clinical isolates fail to grow on the media used for the determination of X and V factor requirement. Nearly all recent isolates from developing countries produce β-lactamase.

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[1] Van Dyck E, Meheus AZ, Piot P. *Laboratory diagnosis of sexually transmitted diseases*. Geneva, World Health Organization, 1999.

[2] Mueller–Hinton agar base, supplemented with 5% sterile horse blood heated to 75 °C, 1% IsoVitaleX, and 3 g/ml vancomycin.

Purulent exudates, wounds, and abscesses

Introduction

One of the most commonly observed infectious disease processes is the production of a purulent (sometimes seropurulent) exudate as the result of bacterial invasion of a cavity, tissue, or organ of the body. Such infections may be relatively simple, innocuous "pimples" or a series of multiple pockets of pus found in abscesses in one or more anatomical sites. The exudate consists of white blood cells, predominantly polymorphonuclear leukocytes, the invading organisms, and a mixture of body fluid and fibrin. In some instances, the exudate may be found as a coating on the surface of an organ, e.g. the surface of the brain in acute bacterial meningitis. In other cases, the exudate may be walled off by layers of fibrin and a network of tissue cells, e.g. a carbuncle or subcutaneous "boil", while in still other cases the exudate may be associated with an open wound, which therefore drains thick fluid or pus.

Just as the anatomical site of exudate production can vary considerably, so too can the organisms involved in the underlying infection. All bacteria that are part of the normal flora, or that gain access to the body, may be involved in the production of an exudate. Some fungi, particularly those that are able to multiply in body tissues, can also be involved in the production of an exudate. In contrast, a purulent exudate is rarely produced in a viral infection.

The bench microbiologist should be aware of the diversity of anatomical sites and microorganisms involved, and be prepared to make the appropriate macroscopic and microscopic examinations and the proper primary media inoculations to recover the major organism(s) involved. Once the organisms have been isolated in pure culture, the identification process and antimicrobial susceptibility tests should be set up as soon as possible.

Communication between the clinician and the microbiologist is particularly important in the diagnosis and management of patients with suppurative infectious diseases. The microbiologist must collaborate with the physician to ensure proper specimen collection and the expeditious delivery of the specimen to the laboratory for prompt and proper processing.

Commonly encountered clinical conditions and the most frequent etiological agents

Surgical specimens

Surgical specimens may be obtained by aspiration of a localized abscess or other surgical procedures. The surgeon should be advised to obtain several small representative tissue samples and any purulent exudate. If possible, cotton swabs should be avoided. The exudate should be collected using a needle and syringe. If cotton swabs must be used, as much exudate as possible should be collected and dispensed into appropriate containers for dispatch to the laboratory. Upon receipt, the laboratory should review the information

provided and then plan cultures for the organisms likely to be found in the particular specimen.

A few examples of conditions and organisms found in different types of surgical specimens are given below:

- The *peritoneal cavity* is likely to contain Gram-negative enteric bacteria (Enterococci), Gram-negative anaerobic rods (*Bacteroides fragilis*), and clostridia.
- A walled-off *abscess* may contain any type of organism, either single or multiple species: Gram-positive cocci and Gram-negative bacilli are most frequently isolated. Anaerobic bacteria and amoebae may also need to be considered, depending on the abscess site.
- *Lymph nodes* are frequently involved in systemic infections. They become swollen and often quite tender, and purulent exudate frequently accumulates. If the node is fluctuant, the liquid contents can be aspirated by a physician. Lymph node biopsies or aspirates from children should be cultured for *Mycobacterium tuberculosis* and other mycobacteria. In addition to being cultured for staphylococci, streptococci, and Gram-negative enteric bacteria, lymph nodes may be good specimens for the diagnosis of systemic and subcutaneous mycoses (histoplasmosis, sporotrichosis).
- *Skin* and *subcutaneous tissue* are prime targets for both abscesses and wound infections. As a general rule, subcutaneous abscesses are caused by staphylococci. Open, weeping skin lesions often involve β-haemolytic streptococci and/or staphylococci, as in impetigo. Another variety of skin lesion requiring some surgical intervention, and often seen as a hospital-acquired infection, is decubitus ulcer or bed sore. The bacteria are frequently skin commensals or intestinal flora that have proliferated in the most external part of the ulcer and create an unpleasant odour and appearance. Routine culture of those organisms is not clinically relevant. The organisms most commonly isolated from biopsy tissue are enteric bacilli; the same organisms may be found in cultures of the superficial exudate. It is not always possible to evaluate the role of these organisms in decubitus ulcers, but healing requires that the ulcer be kept clean, dry, and free of bacteria. Occasionally organisms in a decubitus ulcer may find their way into the blood stream, producing serious complications.
- *Burns*, especially second- and third-degree burns, are prone to infection with a variety of bacterial species. It is very important that careful surgical débridement be carried out prior to obtaining material for culture. Staphylococci and *Pseudomonas aeruginosa* are most commonly encountered.
- *Exudate*s. Sometimes a serous or purulent fluid will collect in a cavity that normally possesses a very small amount of sterile fluid, e.g. the pericardial sac, pleural cavity, bursa, or joint. Needle aspiration under aseptic conditions will yield a laboratory specimen from which the infecting organism may be isolated and identified. Bacteria are usually the cause, but fungi or viruses may also be responsible. The infections are usually monospecific, but mixed aerobic and anaerobic infections do occur. Aspirates from the pleural cavity may yield pneumococci, streptococci, *H. influenzae*, anaerobic streptococci, or anaerobic Gram-negative rods (*Prevotella* and *Porphyromonas*) or *M. tuberculosis*.

Penetrating wounds

Any lesion caused by a penetrating object that breaks the skin is likely to contain a mixture of microorganisms; these organisms are generally part of the skin flora or of the normal microbial flora of soil and water. A penetrating

wound involving damage to the intestines will lead to an even greater threat because the intestinal flora may contribute to infection of the wound and peritoneal cavity.

Penetrating or cutting wounds may be caused by sharp or blunt objects. Metal, glass, wood, etc. are frequently responsible for penetrating wounds, whether caused by accident or deliberately (e.g. stab or gunshot wounds). Tetanus resulting from a penetrating wound is a life-threatening disease in a non-immunized individual. Similarly, wound botulism may go undiagnosed if the physician and the microbiologist are not aware of this possibility. The diagnoses of tetanus and botulism are best made clinically, and laboratory support should be provided by a central reference laboratory. People working with animals or their products are at risk of infection with spores of *Bacillus anthracis*, which may gain access through small wounds or skin abrasions and produce the typical black eschar of anthrax. Other soil organisms, such as *Clostridium perfringens*, may be involved in deep penetrating wounds and give rise to gas gangrene.

Animal bites or scratches occur frequently in both urban and rural areas. The bite may be from a domestic pet, a farm animal, or a wild animal. Rabies must be the prime and immediate concern. Once the possibility of rabies has been eliminated, the other possible etiological agents are many and varied. Rabies diagnosis is too specialized to be discussed in this manual.[1]

The mouths of all animals contain a heterogeneous flora consisting of aerobic and anaerobic bacteria, yeasts, protozoa, and viruses. Infections resulting from bites or scratches are predominantly caused by bacteria. A prime example is infection by *Pasteurella multocida*, which often follows a dog or cat bite if the bite has not been properly cleansed and treated. Human bites may sometimes result in a serious mixed infection of aerobic and anaerobic bacteria.

Nosocomial wound infections

One of the main concerns in the care and treatment of hospitalized patients is that they should not be harmed in the course of the diagnosis and treatment of their illness. Unfortunately, 5–10% of hospitalized patients do acquire an infection while in hospital. Nosocomial infections are costly and can usually be avoided or greatly reduced. Many hospital-acquired infections are known to occur in surgical departments. The rate of postoperative wound infection varies from hospital to hospital, and within a given hospital is likely to be highest in patients who have undergone abdominal, thoracic, or orthopaedic surgery. Surgical wound infections may occur shortly after surgery or several days postoperatively. The site of infection may be limited to the suture line or may become extensive in the operative site. *Staphylococcus aureus* (usually benzylpenicillin-resistant, and now often meticillin-resistant) is the biggest offender, followed closely by *E. coli* and other enteric bacteria. Anaerobic bacteria from the patient's large bowel may gain access to the operative site, making a mixed infection a serious and fairly frequent occurrence in hospitals in which the postoperative wound care and infection prevention programmes are weak. *Bacteroides fragilis* and, occasionally, *Clostridium perfringens* may invade the bloodstream, resulting in a systemic and frequently fatal postoperative infection.

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[1] Meslin F-X, Kaplan MM, Koprowski H, eds. *Laboratory techniques in rabies*, 4th ed. Geneva, World Health Organization, 1996.

An infrequent but challenging infection may follow dental or oral surgery, when a sinus tract from the inside works its way to the skin surface on the face or neck and the discharge contains the "sulfur" granules of actinomycosis.

Collection and transportation of specimens

It is not possible, here, to describe in detail the procedures for specimen collection from each type of wound, abscess, etc. It should be obvious that this is a task that requires close cooperation between the laboratory and the physician. In many instances, there is only one opportunity to obtain a specimen; second specimens are unavailable in many cases. Therefore, proper collection, transport, and storage of these specimens are of the greatest importance, and compromises should be avoided. Once a specimen has been obtained, packaged, and dispatched to the laboratory, it should be processed as soon as possible. After the preliminary examinations have been completed and the cultures made, the rest of the specimen should be properly labelled, stoppered, and refrigerated, until it is certain that no additional laboratory tests are needed.

Abscess

Once an abscess, or multiple abscesses, have been found, the physician or surgeon and the microbiologist should consult on what is to be done. The technique for collecting pus and pieces of the abscess wall is a surgical procedure. A syringe and needle are used to aspirate as much as possible of the purulent material, which is then aseptically transferred to sterile specimen containers. If such containers are not available, the specimen should be kept in the syringe with the needle capped, and the syringe itself should be transported to the laboratory. This material should be processed immediately by the laboratory; both aerobic and anaerobic cultures can be made from a single specimen.

A similar situation exists when the surgeon encounters one or more walled-off abscesses in an organ, or in the thorax, abdomen, or pelvis, during the course of a surgical procedure for another purpose. In anticipation of this, the laboratory should arrange to have stored, in the sterile surgical supplies, a kit for obtaining the contents of such abscesses so that specimens can be delivered promptly to the laboratory for processing. Every effort should be made to avoid the use of swabs for collecting a small amount of specimen, when a large amount is actually present. A swab can justifiably be used to collect very small amounts of pus, or pus from sites that require care, e.g. from the eye. When pieces of tissue are obtained from the abscess wall, the laboratory technician should either grind the tissue, using a small amount of sterile broth as a diluent, or mince the tissue into very small pieces using sterile scissors. Aerobic and anaerobic cultures should be prepared as indicated on pages 92–93.

Infected lacerations, penetrating wounds, postoperative wounds, burns, and decubitus ulcers

No standard procedure for specimen collection can be formulated. However, certain fundamental guidelines should be followed to obtain the best possible specimen for laboratory analysis. After carefully cleaning the site, the surgeon should look beneath the surface for collections of pus, devitalized

tissue, the oozing of gas (crepitation), or any other abnormal sign. Segments of the tissue involved that are to be used for cultures should be removed and placed on sterile gauze for processing. Pus or other exudate should be carefully collected and placed in a sterile tube. Swabs may be used if necessary.

Sinus tract or lymph node drainage

When a sinus tract or lymph node shows evidence of spontaneous drainage, the drainage material should be collected carefully, using a sterile Pasteur pipette fitted with a rubber bulb, and placed in a sterile tube. If discharge is not evident, the surgeon should obtain the purulent material using a sterile syringe and needle or probe. Again, swabs should only be used if a sterile Pasteur pipette is not available.

Exudates

The abnormal accumulation of fluid within a body cavity such as the pleural space, a joint, or the peritoneal space, requires a surgical procedure to aspirate the accumulated material into a sterile container for subsequent delivery to the laboratory for microbiology and cytology. In those cases where the accumulation persists and an open drain is put in place, it is necessary to collect the drainage fluid in an aseptic manner for subsequent culture and other tests.

Macroscopic evaluation

Specimens of pus or wound discharge collected on swabs are difficult to evaluate macroscopically, particularly when the swab is immersed in a transport medium. Specimens of pus, received in a syringe or in a sterile container, should be evaluated carefully by an experienced technician for colour, consistency, and odour.

Colour

The colour of pus varies from green-yellow to brown-red. A red colour is generally due to admixture with blood or haemoglobin. The aspirate from a primary amoebic liver abscess has a gelatinous consistency and a dark brown to yellowish brown colour. Pus from postoperative or traumatic wounds (burns) may be stained blue-green by the pyocyanin pigment produced by *Pseudomonas aeruginosa*.

Consistency

The consistency of pus may vary from a turbid liquid to one that is very thick and sticky. Exudates, aspirated from a joint, the pleural cavity, the pericardial sac, or the peritoneal cavity, are generally liquid, with all possible gradations between a serous exudate and frank pus.

Pus originating from a draining sinus tract in the neck should be inspected for small yellow "sulfur" granules, which are colonies of the filamentous *Actinomyces israelii*. The presence of sulfur granules suggests a diagnosis of cervicofacial actinomycosis. Small granules of different colours (white, black, red, or brown) are typical of mycetoma, a granulomatous tumour, generally involving the lower extremities (e.g. madura foot), and characterized by mul-

tiple abscesses and draining sinuses. The coloured granules correspond to either filamentous bacteria or fungal mycelium.

Pus from tuberculous "cold abscesses" (with few signs of inflammation) is sometimes compared with soft cheese and called "caseum" or "caseous pus".

Odour

A foul feculent odour is one of the most characteristic features of an anaerobic or a mixed aerobic–anaerobic infection, although it may be lacking in some instances. The odour, together with the result of the Gram-stained smear, should be reported at once to the clinician as it may be helpful in the empirical selection of an appropriate antimicrobial. It will also help in determining whether anaerobic cultures are needed.

Microscopic examination

A smear for Gram-staining and examination should be made for every specimen. In particular cases, or at the request of the clinician, a direct wet mount may be prepared and Ziehl–Neelsen staining carried out.

Gram-stained smear

Using a bacteriological loop, make an even smear of the most purulent part of the specimen on a clean slide. If only a swab is available, the slide should first be sterilized by being passed through the flame of a Bunsen burner and allowed to cool. The cotton swab should then be gently rolled over the glass surface, without rubbing or excessive pressure. Allow the slide to air-dry, protected from insects, or place it in the incubator. Fix by heat, stain and examine the smear under the oil-immersion objective (×100). Inspect carefully and note the presence and the quantity (use +signs) of:

— polymorphonuclear granulocytes (pus cells);
— Gram-positive cocci arranged in clusters, suggestive of staphylococci;
— Gram-positive cocci in chains, suggestive of streptococci or enterococci;
— Gram-negative rods resembling coliform (*Escherichia coli*, *Klebsiella*, etc.), other Enterobacteriaceae (*Proteus*, *Serratia*, etc.), nonfermentative rods (*Pseudomonas* spp.), or obligate anaerobes (*Bacteroides* spp.);
— large straight Gram-positive rods with square ends suggestive of *Clostridium perfringens*, the principal agent of gas gangrene, or *Bacillus anthracis*, the agent of anthrax;
— an extremely heavy and pleomorphic mixture of bacteria, including streptococci, Gram-positive and Gram-negative rods of various sizes, including fusiform rods; such a picture is suggestive of "mixed anaerobic flora" and should be reported as such;
— *Candida* or other yeast cells, which are seen as ovoid Gram-positive budding spheres, often forming branched pseudomycelia.

Sulfur granules from actinomycosis or granules from a mycetoma should be crushed on a slide, Gram-stained and inspected for thin branched and fragmented Gram-positive filaments.

Direct microscopy

When requested, or when a fungal or parasitic infection is suspected, a wet preparation should be examined. If the pus is thick, a loopful should be mixed in a drop of saline. When looking for fungi, a drop of 10% potassium hydroxide should be used to clear the specimen. Apply a coverslip and, using the ×10 and ×40 objectives, look especially for:

— actively motile amoebae in aspirate from a liver abscess;
— yeast cells of *Histoplasma capsulatum* (including the African var. *duboisii*,) *Blastomyces dermatitidis* (in endemic areas), *Candida* spp.;
— fungal hyphae and bacterial filaments in crushed granules from mycetoma;
— parasites, such as microfilariae, scolices or hooks of *Echinococcus*, eggs of *Schistosoma*, *Fasciola*, or *Paragonimus*.

Acid-fast staining (Ziehl–Neelsen)

Ziehl–Neelsen staining should be performed when requested by the clinician. It is also advisable to make an acid-fast stained preparation when the pus shows no bacteria or when only faintly stained Gram-positive "coryneform" rods are seen on the Gram-stained smear. Tubercle bacilli should be suspected, in particular, in pus or purulent exudate from the pleura, joints, bone abscesses, or lymph nodes. Non-tuberculous (so-called "atypical") acid-fast bacilli are sometimes found in gluteal abscesses at the site of deep intramuscular injections. Such abscesses are often caused by rapidly growing mycobacteria belonging to the *Mycobacterium fortuitum–chelonei* group. In the tropics, discharge scraped from the base of a necrotic skin ulcer situated on a leg or an arm may be due to slow-growing acid-fast rods called *M. ulcerans* (Buruli ulcer). *M. marinum* is another tuberculous acid-fast rod, which may be found in chronic, ulcerative, nodular lesions on the hands, arms, and other exposed skin surfaces in swimmers and fishermen.

Culture

If bacteria or fungi are seen on microscopic examination, appropriate culture media should be inoculated. Independently from the results of microscopy, all specimens of pus or exudate should preferably be inoculated onto a minimum of three culture media:

— a blood agar plate for the isolation of staphylococci and streptococci;
— a MacConkey agar plate for the isolation of Gram-negative rods; and
— a tube of broth that can serve as enrichment medium for both aerobes and anaerobes, e.g. thioglycollate broth or cooked meat medium.

The size of the inoculum should be determined according to the result of the microscopic examination, and may vary from one loopful to a few drops. If massive numbers of organisms are seen on the Gram-stained smear, the specimen may even have to be diluted in a small amount of sterile broth before plating out. If a swab is used for the inoculation, it should be applied to a small area of the plate and the rest of the surface streaked out with a loop. If the swab is dry, it should first be moistened in a small quantity of sterile broth or saline. In all cases, the technique of inoculation should provide single colonies for identification and susceptibility tests.

Prior to inoculation, the blood agar plate should be dried for 20 minutes in an incubator to minimize the risk of overgrowth by spreading *Proteus* spp. The inoculated plate should be incubated at 35 °C in a candle jar. Routinely, all media should be incubated for two days and inspected daily for growth. If culture for fastidious organisms is requested, longer incubation (1–2 weeks or more) will be necessary. If growth appears in the broth, it should be Gram-stained and subcultured onto appropriate culture media. Additional culture media should be used if specially requested, or if indicated by the results of the microscopic examination, as in the following examples:

- If staphylococci have been seen, an additional mannitol salt agar is helpful in obtaining pure growth and in making a preliminary distinction between *S. aureus* and other cocci.
- If streptococci have been observed, their identification may be hastened by placing a differential bacitracin disc on the initial streaking area.
- If yeasts or fungi have been observed, the specimen should also be inoculated onto two tubes of Sabouraud dextrose agar, one to be incubated at 35 °C, the other at room temperature, both to be observed for up to one month. (Blood agar is sufficient for the isolation of *Candida* spp.)
- If acid-fast rods have been seen in the Ziehl–Neelsen stained smear, up to 3 tubes of Löwenstein–Jensen medium should be inoculated. If the specimen also contains non-acid-fast bacteria, it should first be decontaminated. Rapidly growing mycobacteria, such as *M. fortuitum*, may be killed by the decontamination process; they produce growth within 3–7 days on blood agar and MacConkey agar. Branched, filamentous, partially acid-fast rods in pus from the pleura or from a brain abscess will probably be *Nocardia asteroides*, which grows on blood agar within a few days.
- Pus from patients with arthritis, pleuritis, osteitis, or cellulitis, particularly from children under 5 years of age, should also be inoculated onto a chocolate agar plate for the recovery of *H. influenzae*.
- Culture in a strictly anaerobic atmosphere is essential when the Gram-stained smear shows mixed anaerobic flora and also when the specimen produces a typical foul odour. Anaerobic blood agar is also necessary for the growth of *Actinomyces*. Anaerobic culture will be requested by the clinician when he or she suspects clostridial gas gangrene. Methods for anaerobic bacteriology are described on pages 98–102.

Identification

With the exception of contaminants from the environment or from the skin (such as *Staphylococcus epidermidis*), all organisms isolated from wounds, pus, or exudates should be considered significant and efforts made to identify them. Full identification, however, is not always necessary, particularly in the case of mixed flora.

Bacteria and fungi isolated from pus and exudates may belong to almost any group or species. Identification criteria are given here only for staphylococci commonly associated with pus (pyogenic), and for two other pathogens, *Pasteurella multocida* and *Bacillus anthracis*, which are rarely isolated from wounds or skin infections, but are very important for the management of the patient. A standard textbook of clinical microbiology should be consulted for a full description of identification methods. In every case, the first step should be to examine well-separated colonies carefully, pick a single colony of each type, prepare a Gram-stained smear, and then characterize the organisms under the microscope.

Staphylococci

Staphylococci are the bacteria most frequently associated with the production of pus. Staphylococci grow well aerobically on blood agar and form opaque white to cream colonies, 1–2 mm in diameter, after overnight incubation. They are unique in growing on media with a high salt content, such as MSA. They can be differentiated from streptococci by their morphology and by the production of catalase. Catalase production by staphylococci is shown by placing it in a drop of 3% hydrogen peroxide onto the colonies deposited on a clean glass slide. The appearance of bubbles of oxygen is an indication of catalase production.

For clinical purposes, staphylococci can be divided into those that produce coagulase and those that do not. The coagulase-producing staphylococci belong to the species *S. aureus*, which is the species of greatest medical interest. Of the several coagulase-negative species, only two will be considered here—*S. epidermidis* and *S. saprophyticus*.

Although *S. aureus* is part of the commensal microbial flora of the nose (40% of healthy adults are positive), skin, and intestinal tract, this species causes impetigo, boils, abscesses, wound infection, infection of ulcers and burns, osteomyelitis, mastitis (breast abscess), pleural empyema, pyomyositis, toxic shock syndrome, and other types of pyogenic infection.

S. epidermidis is also a common commensal of the skin, nose, and other mucous membranes and possesses a very low pathogenicity. However, its presence in pus should not always be dismissed as skin contamination. Despite its low infectivity, *S. epidermidis* can cause skin infections at the site of an in-dwelling catheter, cannula, or other device. Infections with *S. epidermidis* are particularly troublesome in cardiac and orthopaedic surgery involving the insertion of prosthetic devices (artificial heart valves or artificial hips).

S. saprophyticus is recognized to be a common cause of urinary tract infections in young women, being second only to *E. coli* in some populations.

The distinctive features of the three main species of Staphylococcus are given in Table 22. A flow diagram for the preliminary identification of staphylococci is shown in Fig. 10.

In view of the importance of the coagulase test in the identification of *S. aureus*, this test is described here in detail. Coagulase is an enzyme that causes plasma to clot. Staphylococcal coagulase exists in two forms: bound coagulase or

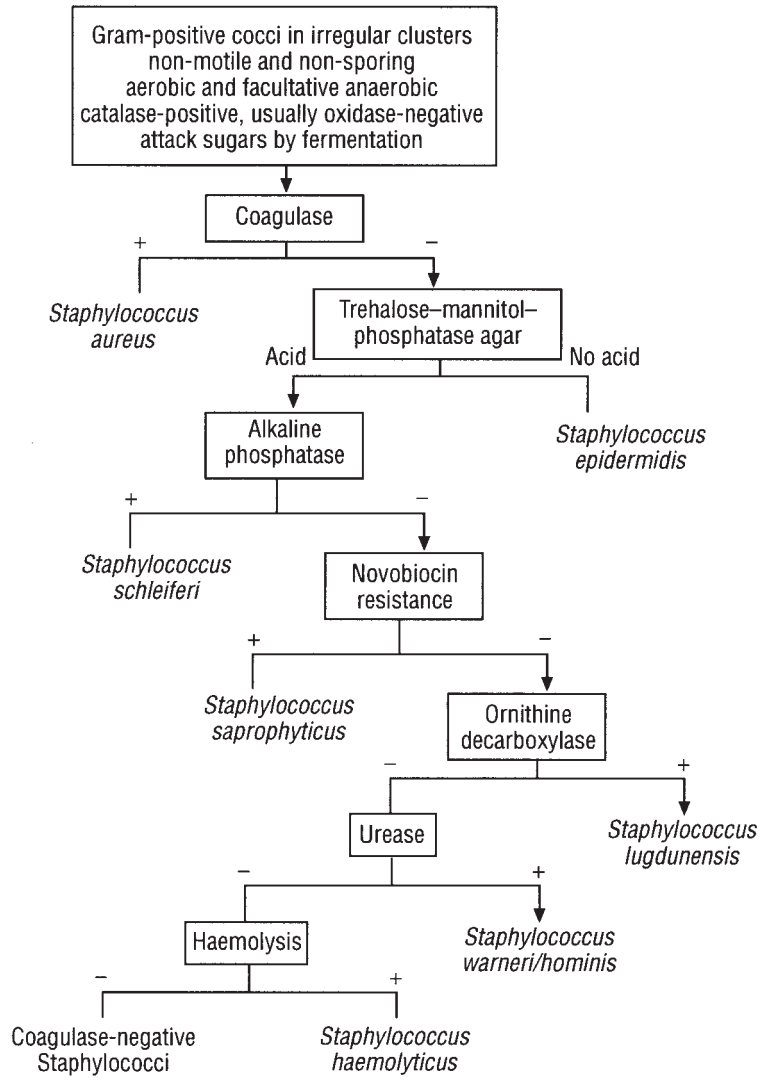
Table 22. Differentiation of medically important species of *Staphylococcus*

	S. aureus	***S. epidermidis***	***S. saprophyticus***
Production of coagulase	yes	no	no
Acidification of mannitol on mannitol salt agar[a]	acid (yellow)	neutral (red)	acid (yellow)
Pigment of colonies[a]	grey, cream or yellow	white	white
In vitro susceptibility to novobiocin	susceptible	susceptible	resistant[b]
DNase agar	yes	no	no

[a]Exceptions are possible.

[b]Inhibition zone of less than 16 mm, using a 5-μg disc in the standardized disc-diffusion method.

Fig. 10. Flow diagram for the preliminary identification of human *Staphylococcus* species



clumping factor, which is demonstrated in the slide test, and free coagulase, which is demonstrated in the tube test.

- **Slide test.** On a clean slide, emulsify one or a few similar colonies of staphylococci in a drop of saline. The suspension must be fairly thick. Dip a straight wire into plasma and use this to stir the bacterial suspension. Observe for clumping within 10 seconds. False-negative slide tests occur approximately 10% of the strains of *S. aureus*. If the slide test is negative for an isolate that seems to be pathogenic on other grounds (pigment, clinical source), it should be re-examined in the tube test.
- **Tube test.** Dispense a few drops (0.5 ml) of plasma into a sterile 12 × 75 mm tube, and add two drops of the pure culture in broth. A suspension with an equivalent density may also be prepared directly from growth on blood agar. Incubate the tube at 35 °C for 4–18 hours and then examine for clotting.

The plasma used in the coagulase test may be fresh human or rabbit plasma obtained with ethylenediamine tetraacetic acid (EDTA). It should be stored in

the refrigerator in small amounts (1 ml), and its performance checked with cultures of *S. aureus* and *S. epidermidis*, run in parallel.

Pasteurella multocida

A number of Gram-negative bacilli are transmitted by animal bites and can cause severe infections in human beings, the most prevalent being *Pasteurella multocida*. *P. multocida* is a commensal found in the normal mouth flora of many animals. A bite wound infected with *P. multocida* may give rise to extensive cellulitis, which may extend to a joint, producing arthritis. Osteomyelitis, bacteraemia, and even meningitis have been described.

P. multocida should be looked for specifically in wound discharge if an animal bite is known or suspected of being the cause of the wound. *P. multocida* is a very small, Gram-negative, nonmotile coccobacillus. It grows well on blood agar at 35 °C but is completely inhibited by the bile salts contained in the enteric selective media, e.g. MacConkey agar. After overnight incubation, the colonies on blood agar are small, nonhaemolytic, translucent, and mucoid (due to the presence of a capsule in the virulent form).

Biochemical identification is based on the following characteristics:

— fermentation of glucose without gas: *P. multocida* grows on Kligler iron agar with acidification of the butt;
— oxidase test is weakly positive;
— catalase-positive;
— nitrate reduced to nitrite (0.1% potassium nitrate in nutrient broth);
— urease-negative;
— indole-positive—test in tryptic soy broth (TSB) or on MIU after 48 hours incubation;
— highly sensitive to benzylpenicillin in the disc-susceptibility test.

Bacillus anthracis

The genus *Bacillus* is composed of numerous species of aerobic, spore-forming, Gram-positive rods, widely distributed in the soil. The species *B. anthracis* is of public health importance in skin infections. The other species, when isolated from wounds or pus, are generally contaminants or, at most, opportunists.

B. anthracis is a major pathogen of cattle, sheep, goats, and other domestic animals. It also affects wild animals. Anthrax can affect human beings, particularly people in parts of Africa and Asia working or living in close contact with livestock. Human infection may also derive from animal products containing anthrax spores, such as wool, skins, fur, and bones.

The commonest form of human infection is cutaneous anthrax, which may progress to septicaemia and meningitis. The spore enters through damaged skin and produces a vesicular lesion with a necrotic centre, surrounded by extensive oedema ("malignant pustule"). Large Gram-positive, square-ended, capsulated rods without spores are seen in smears from the vesicular fluid.

B. anthracis grows aerobically. On blood agar it produces large, flat, greyish colonies, up to 5 mm in diameter, with a rough surface texture and irregular edges showing hairy outgrowths (Medusa head). It is important at this stage

to differentiate the highly pathogenic *B. anthracis* from the generally harmless saprophytic species.

The preliminary distinction should be based on the absence of haemolysis, benzylpenicillin sensitivity, and the lack of motility in *B. anthracis*. In contrast, most of the saprophytic *Bacillus* species are motile and strongly haemolytic. These three features can form the basis of a presumptive identification. For definitive diagnosis, a pure culture of the isolate should be sent immediately to the central veterinary or public health laboratory.

B. anthracis is a highly infectious organism and specimens and cultures should be handled with the greatest care to avoid contamination of the environment and infection of laboratory personnel.

Susceptibility testing

Antimicrobials may not always be needed for the management of patients with wounds, abscesses, or exudates. Proper surgical incision, drainage and débridement are generally more important than antimicrobial drugs. The results of susceptibility tests should be made available, however, within 48 hours after receiving the specimen.

Routine susceptibility tests should not be performed on bacteria that have a known sensitivity pattern, such as streptococci, *Pasteurella*, and *Actinomyces*, which are almost without exception susceptible to benzylpenicillins.

For Enterobacteriaceae, non-fermentative Gram-negative rods, and staphylococci, the standardized disc-diffusion test (Kirby–Bauer) should be used. Only antimicrobials currently being used by the requesting physicians should be tested. New and expensive antimicrobials should only be tested (or reported) on special request, or when the isolate is resistant to other drugs.

Problems are often encountered when testing the susceptibility of staphylococci, both *S. aureus* and *S. epidermidis*. Over 80% of isolates, even from the community, produce β-lactamase and are resistant to benzylpenicillin and ampicillin. Infections caused by benzylpenicillin-resistant staphylococci are often treated with β-lactamase-resistant penicillins of the meticillin-group (oxacillin, cloxacillin, etc.). The oxacillin disc (1 µg) is currently recommended for testing the susceptibility to this group. Oxacillin discs are stable, and apt to detect resistance to the whole group. This resistance is often of the heteroresistant type, i.e. it involves only a part of the bacterial population. As heteroresistance of staphylococci is easier to recognize at low temperatures, the incubator temperature should not exceed 35 °C. A heteroresistant strain shows, within an otherwise definite zone of inhibition, a film of hazy growth or numerous small colonies that are often dismissed as contaminants. If such growth appears, a Gram-stained smear is indicated to exclude contamination.

Heteroresistant strains are clinically resistant to all the β-lactam antimicrobials including cefalosporins, carbapenems, and the meticillin group. For this reason staphylococci need not be tested for susceptibility to cefalosporins. There is complete cross-resistance between benzylpenicillin and ampicillin. Staphylococcal susceptibility to ampicillin should therefore not be tested separately.

Anaerobic bacteriology

Introduction

This manual frequently refers to anaerobic bacterial infections and anaerobic bacteria. The former may occur in virtually any body tissue and at any body site provided that the prevailing conditions are suitable.

The majority of anaerobic bacterial diseases are caused by endogenous bacteria that are part of the normal body flora and that are perfectly compatible with health until something happens to disturb the balance of the normal flora, or to allow the passage of bacteria from one anatomical site to another. Exogenous anaerobic bacteria, primarily *Clostridium tetani*, *C. botulinum*, and occasionally *C. perfringens* and other clostridial species, can gain access through wounds, causing tetanus, wound botulism, or gas gangrene. Abscesses of practically any organ, bacteraemia, peritonitis, thoracic empyema, cellulitis, and appendicitis are just a few conditions in which anaerobic bacteria may play a very significant role in the disease process. It is therefore important that the microbiologist knows when and how to culture for anaerobic bacteria in a given clinical specimen.

Description of bacteria in relation to oxygen requirement

A perhaps over-simplistic, but operationally acceptable, description of medically important bacteria in relation to their oxygen requirements is as follows:

- *Obligate aerobic bacteria* require gaseous oxygen to complete their energy-producing cycle; these organisms cannot grow without a source of oxygen. Examples of obligate aerobic bacteria are *Micrococcus* spp. and *Nocardia asteroides*.
- *Obligate anaerobic bacteria* do not require oxygen for metabolic activity, and in fact oxygen is toxic to many of them. Energy is derived from fermentation reactions, which may produce foul-smelling end-products. Examples of such anaerobic bacteria are *Bacteroides fragilis* and *Peptostreptococcus magnus*.
- *Facultative anaerobic bacteria* are those for which there is no absolute requirement for oxygen for growth or energy production; they can either utilize oxygen or grow by anaerobic mechanisms. Such bacteria are most versatile, and are usually able to adapt to their environment, creating energy for growth and multiplication by the most effective mechanism. *E. coli* and *S. aureus* are examples of facultative anaerobic organisms.

There are, in addition to the above, *microaerophilic bacteria* that grow best at reduced oxygen tensions. *Campylobacter jejuni* is an example of a microaerophilic bacterium.

Bacteriology

Four major groups of anaerobic bacteria account for approximately 80% of the diagnosed anaerobic infections. These are *Bacteroides*, *Prevotella* and *Porphyromonas* spp., *Peptostreptococcus* spp., and *Clostridium* spp. The most

frequently encountered species in each genus are: *Bacteroides fragilis*, *Peptostreptococcus magnus*, and *Clostridium perfringens*. Specific methods for the isolation and identification of these three genera and species should also serve as a model for isolating and initiating the identification process of other clinically important anaerobic bacteria.

Specimen collection and transport to the laboratory

This has been covered throughly in previous sections. Swabs are to be avoided for the collection of specimens, as anaerobes are very sensitive to air and drying. Specimens for the culture of anaerobes should be taken carefully from the active site of infection. The services of a surgeon may be required for the collection of some specimens. This is particularly true of needle aspiration of pus, obtaining tissue and/or pus specimens from infected wounds, empyema, or draining abscesses.

The specimen should be placed in a sterile, tightly closed container, or if one of these is not available, the entire aspirated specimen should be transported immediately to the laboratory in the syringe, with the needle capped or protected by a rubber stopper.

Establishing an anaerobic environment for incubating cultures

A variety of methods exist for creating an anaerobic environment. One that is simple and inexpensive is the use of an anaerobic jar made of thick glass or polycarbonate, with a capacity of 2.5–3.5 litres, that is equipped with a secure gas-proof lid which can be easily removed and replaced. After putting the inoculated Petri dishes into the jar, an anaerobic atmosphere is generated by introducing a commercially available disposable anaerobiosis-generating device, and closing the lid. Disposable devices for generating anaerobiosis take the form of flat, sealed, foil envelopes that release hydrogen and carbon dioxide after addition of water. These devices, however, require a palladium catalyst fixed on the underside of the lid of the jar. The catalyst becomes inactivated during use and should be regenerated or replaced at regular intervals, as recommended by the manufacturer. Disposable redox-indicator strips, which change from blue (or red) to colorless in an anaerobic atmosphere, are available from a number of manufacturers.

Tubes of broth cultures for anaerobes, such as thioglycollate broth or cooked meat broth, need not be incubated under anaerobic conditions, because their formulations contain reducing substances that will create an anaerobic environment. When the volume of broth is sufficient (10–12 ml per 15-mm diameter standard screw-top tube) and the medium freshly prepared, anaerobic conditions are produced in the lower part of the tube. If not used on the day of preparation, the tubes should be regenerated with the screw-top loosened for about 15 minutes in a boiling water-bath to remove dissolved oxygen, the screw-top tightened, and the medium allowed to cool before inoculation.

Anaerobic culture media

Anaerobic cultures should be performed only when requested by the clinician, when the specimen has a foul smell, or when the result of the Gram-stained smear indicates the possibility of an anaerobic infection, e.g.

the presence of a mixed pleomorphic flora of Gram-positive and Gram-negative rods and cocci, the presence of Gram-negative fusiform rods, or the presence of square-ended thick Gram-positive rods that may be *Clostridium*.

Routine anaerobic cultures should not be done on urine, genital secretions, faeces, or expectorated sputum; the presence of anaerobic bacteria in these specimens is indicative of contamination with the normal commensal flora of the respective specimen site. Clinicians should be informed that specimens containing normal flora are not acceptable for anaerobic cultures unless there is strong justification.

Ordinary blood agar is a good plating medium for isolation of the most important anaerobic pathogens. For isolation of the more fastidious species, a blood agar base enriched with growth factors (haemin and menadione) is recommended. Such a base is commercially available as Wilkins–Chalgren anaerobe agar.

Anaerobic bacteria are often part of a complex microflora, also involving aerobic organisms, and anaerobic blood agar may be made selective by the addition of one or more specific antimicrobials. For example, the addition of an aminoglycoside (neomycin, kanamycin) in a final concentration of 50 µg/ml inhibits the majority of aerobic and facultative bacteria. A solution of the aminoglycoside is prepared by dissolving 500 mg in 100 ml of distilled water. Melt 100 ml of anaerobic agar base and when it has cooled to 56 °C, add aseptically 5 ml of defibrinated blood and 1 ml of the antibiotic solution. Mix well, and aseptically pour about 15–18 ml into sterile Petri plates. These plates should be used as soon as possible, or kept in a refrigerator, preferably in a plastic bag or sleeve.

Inoculation and isolation procedures

Specimens from suspected anaerobic infections should be inoculated without delay onto the following media:

— an anaerobic blood agar to be incubated in an anaerobic jar;
— an aerobic blood agar to be incubated in a candle jar;
— a plate of MacConkey agar;
— a tube of anaerobic broth (thioglycollate or cooked meat).

The aerobic cultures should be inoculated and processed as usual and examined after 24 and 48 hours for aerobic and facultative organisms. A small area of the anaerobic blood agar plate should be inoculated and the inoculum streaked out with a loop. The plates should be incubated and the anaerobic jar opened after 48 hours for inspection. If growth is insufficient, the plates may be reincubated for a further 24 or 48 hours. The broth culture should be heavily inoculated with a Pasteur pipette so as to distribute the inoculum throughout the medium in the tube.

After 48 hours, the growth on the anaerobic blood agar should be inspected and compared with the growth on the aerobic plating media. Each colony type should be examined with Gram stain. Bacteria with the same microscopic appearance that grow on aerobic and anaerobic agar are considered to be facultative anaerobes. Colonies that appear only on anaerobic agar are probable anaerobes and should be subcultured on two blood agar plates, one to be incubated anaerobically and one in a candle jar. If growth appears only in anaerobiosis, identification of a pure culture of the anaerobe should be attempted.

If growth is observed in the depths of anaerobic broth, it should be subcultured to aerobic and anaerobic blood agar and examined in the same way as the primary culture plates. As the liquid culture is inoculated with a larger volume of pus, it may be positive when the primary plates remain sterile.

Identification of medically important anaerobes

Bacteroides fragilis group

This group includes several related species that belong to the normal flora of the intestine and the vagina. They are frequently involved in abdominal and pelvic mixed infections and may also cause bacteraemia. *B. fragilis* is a non-motile Gram-negative rod, often showing some pleomorphism, growing rapidly on anaerobic blood agar. After 48 hours, the colonies are of moderate size (up to 3 mm in diameter), translucent, grey-white, and non-haemolytic. Rapid identification is possible with the bile stimulation test. A pure culture of the suspected organism is inoculated in the depth of two tubes of thioglycollate broth, with one of the tubes containing 20% (2 ml in 10 ml) sterile (autoclaved) ox bile. After 24 hours of incubation, the growth in the two tubes should be compared: the growth of *B. fragilis* is clearly stimulated in the broth supplemented with bile.

Clostridium perfringens

The genus *Clostridium* contains many species of sporulating Gram-positive rods, some of which belong to the normal gut flora while others are found in dust and soil. The clinically most significant species is *C. perfringens*. It is commonly associated with gas gangrene and may also cause bacteraemia and other deep infections. Unlike most other species of *Clostridium*, it is non-motile and does not form spores in the infected tissues or in young cultures.

C. perfringens grows rapidly in anaerobic broth with the production of abundant gas. On anaerobic blood agar, colonies of moderate size (2–3 mm) are seen after 48 hours. Most strains produce a double zone of haemolysis: an inner zone of complete clear haemolysis, and an outer zone of partial haemolysis.

Rapid identification is possible with the reverse CAMP test,[1] which is performed as follows (see Fig. 11):[2]

1. Prepare a blood agar plate with 5% thrice-washed sheep blood.
2. Streak a pure culture of *Streptococcus agalactiae* along the diameter of the plate. Streak the suspected *Clostridium* culture in a line perpendicular to, but not touching, the *S. agalactiae*.
3. Incubate in an anaerobic jar for 24 hours.

C. perfringens forms an arrowhead-shaped area of enhanced haemolysis at the junction of the two streaks. Reverse-CAMP-negative clostridia may be reported as "*Clostridium* spp. not *C. perfringens*".

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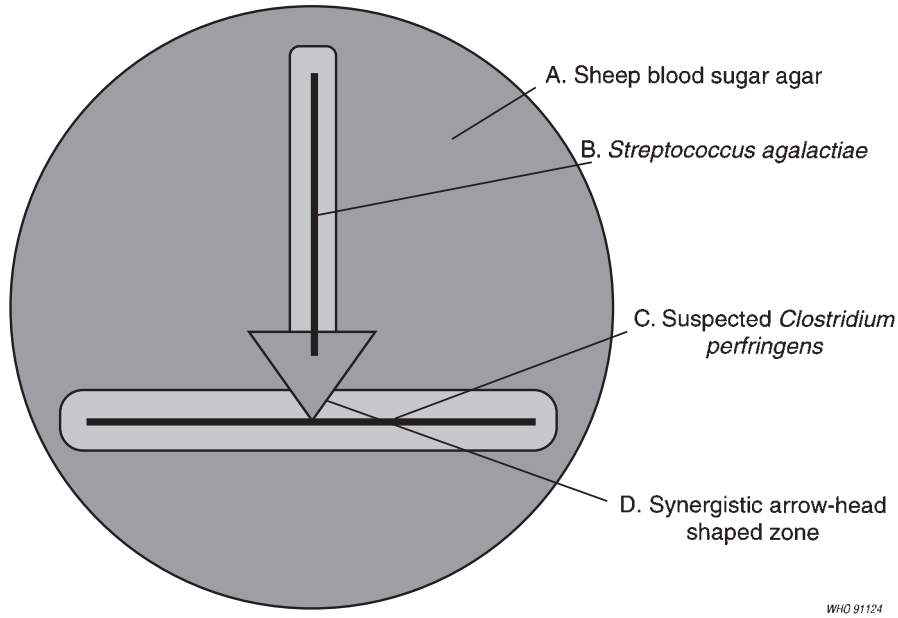
[1] Reverse CAMP test: named after Christy, Adkins and Munch-Peterson who first described this reaction in group B streptococci.

[2] Hansen MV & Elliot LP. New presumptive identification test for *Clostridium perfringens*: reverse CAMP test. *Journal of clinical microbiology*, 1980, **12**:617–619.

Fig. 11. Reverse CAMP test



Peptostreptococcus

Several species of obligate anaerobic Gram-positive cocci belong to the commensal flora of the respiratory, digestive, and urogenital tracts. They are involved, usually in association with other aerobic or anaerobic bacteria, in anaerobic abscesses, wound infections, and even bacteraemia. The growth of anaerobic cocci in laboratory media is usually slower than that of *Bacteroides* or *Clostridium* and colonies are usually not apparent on blood agar until after 48 hours of incubation.

Species identification is not needed in routine bacteriology. Gram-positive cocci that produce small, convex, white colonies on anaerobic blood agar, but do not grow in aerobic conditions, may be presumptively identified as *Peptostreptococcus* spp.

Antimicrobial susceptibility tests

Antimicrobial susceptibility tests should not routinely be performed on anaerobic bacteria, in view of the present lack of agreement on a standardized disc-diffusion test.

Most anaerobic infections are caused by penicillin-sensitive bacteria, with the exception of infections originating in the intestinal tract or the vagina. Such infections generally contain *Bacteroides fragilis*, which produces β-lactamase and is resistant to penicillins, ampicillins, and most cefalosporins. Such infections can be treated with clindamycin, metronidazole, or chloramphenicol. Aminoglycosides and quinolones have no activity against anaerobes, but they are often used for the treatment of patients who have mixed infections, because of their effectiveness against aerobic bacteria, which are often part of the complex flora.

Antimicrobial susceptibility testing

Introduction

At a meeting organized by WHO in Geneva in 1977,[1] concern was expressed about the worldwide increase in antimicrobial resistance associated with the growing, and frequently indiscriminate, use of antimicrobials in both humans and animals. In recent years, drug-resistant bacteria have given rise to several serious outbreaks of infection, with many deaths. This has led to a need for national and international surveillance programmes to monitor antibimicrobial resistance in bacteria by susceptibility testing using reliable methods that generate comparable data. The availability of microbiological and epidemiological information would help clinicians in selecting the most appropriate antimicrobial agent for the treatment of a microbial infection.

If predictions are to be valid, the susceptibility test must be performed by an accurate and reproducible method, the results of which should be directly applicable to the clinical situation. The ultimate criterion of the reliability of any susceptibility testing method is its correlation with the response of the patient to antimicrobial therapy.

The WHO meeting considered that the modified disc-diffusion technique of Kirby–Bauer[2], for which requirements had been established by WHO in 1976, could be recommended for clinical and surveillance purposes in view of its technical simplicity and reproducibility. The method is particularly suitable for use with bacteria belonging to the family Enterobacteriaceae, but it can also be recommended as a general purpose method for all rapidly growing pathogens. It has been adapted to the clinically most important fastidious bacteria but not to strict anaerobes and mycobacteria. It is therefore recommended that the details of this test be made available to laboratory workers.[3]

General principles of antimicrobial susceptibility testing

Antimicrobial susceptibility tests measure the ability of an antimicrobial agent to inhibit bacterial growth in vitro. This ability may be estimated by either the dilution method or the diffusion method.

The dilution test

For quantitative estimates of antimicrobial activity, dilutions of the antimicrobial may be incorporated into broth or agar medium, which is then inoculated with the test organism. The lowest concentration that prevents growth

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[1] *Surveillance for the prevention and control of health hazards due to antibiotic-resistant enterobacteria*. Geneva, World Health Organization, 1978 (WHO Technical Report Series, No. 624).

[2] *WHO Expert Committee on Biological Standardization. Twenty-eighth report*. Geneva, World Health Organization, 1977 (WHO Technical Report Series, No. 610).

[3] A comparable method, based on similar principles and quality control requirements as the Kirby–Bauer method, is the NEO-SENSITABS method, produced by ROSCO Diagnostica, Taastrup, Denmark. This method uses 9-mm colour-coded, antimicrobial tablets, instead of paper discs. The tablet form results in an extraordinary stability with a shelf-life of four years, even at room temperature. This increased stability is very important for laboratories in tropical countries.

after overnight incubation is known as the minimum inhibitory concentration (MIC) of the agent. This MIC value is then compared with known concentrations of the drug obtainable in the serum and in other body fluids to assess the likely clinical response.

The diffusion test

Paper discs, impregnated with a specified amount of an antimicrobial, are placed on agar medium uniformly seeded with the test organism. A concentration gradient of the antimicrobial forms by diffusion from the disc and the growth of the test organism is inhibited at a distance from the disc that is related, among other factors, to the susceptibility of the organism.

There is an approximately linear relation between log MIC, as measured by a dilution test, and the inhibition zone diameter in the diffusion test. A regression line expressing this relation can be obtained by testing a large number of strains by the two methods in parallel (see Fig. 12 and 13).

Clinical definition of terms "resistant" and "susceptible"—the three-category system

The result of the susceptibility test, as reported to the clinician, is the classification of the microorganism in one of two or more categories of susceptibility. The simplest system comprises only two categories: susceptible and resistant. This classification, although offering many advantages for statistical and epidemiological purposes, is too inflexible for the clinician to use. Therefore, a three-category classification is often adopted. The Kirby–Bauer method and its modifications recognize three categories of susceptibility and

Fig. 12. Graphic representation of the relationship between $\log_2$MIC and the inhibition zone diameter obtained by the diffusion test using discs containing a single concentration of antimicrobial

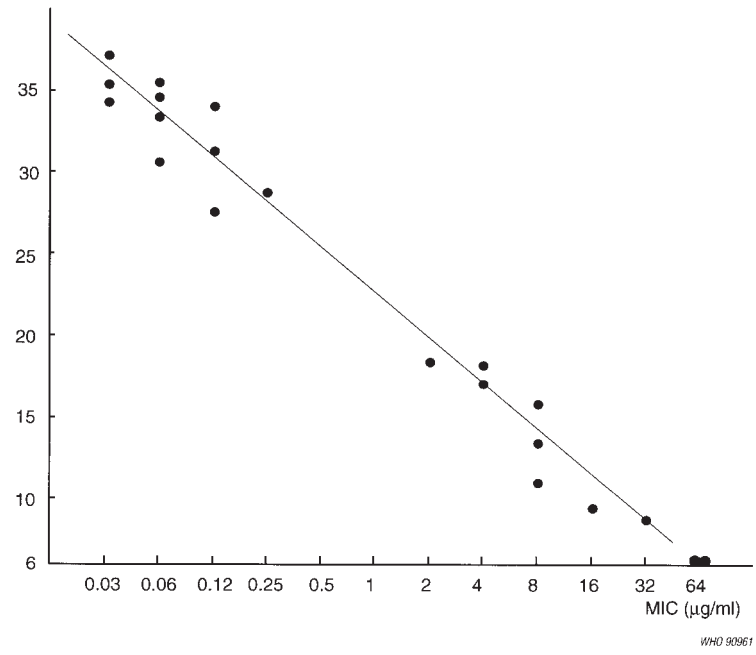
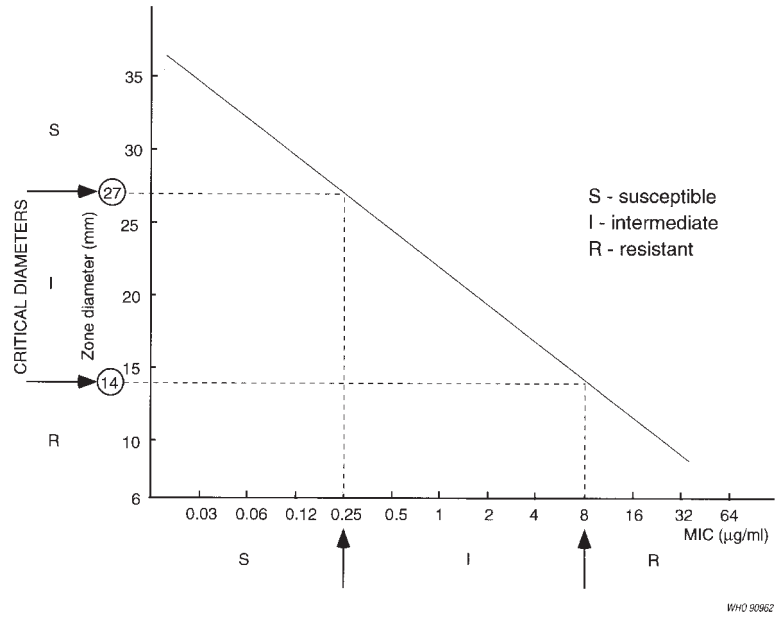


Fig. 13. Interpretation of zone sizes as susceptible, intermediate and resistant by their relationship to the MIC



it is important that both the clinician and the laboratory worker understand the exact definitions and the clinical significance of these categories.

- *Susceptible*. An organism is called "susceptible" to an antimicrobial when the infection caused by it is likely to respond to treatment with this antimicrobial, at the recommended dosage.
- *Intermediate susceptibility* covers two situations. It is applicable to strains that are "moderately susceptible" to an antimicrobial that can be used for treatment at a higher dosage (e.g. β-lactams) because of its low toxicity or because the antimicrobial is concentrated in the focus of infection (e.g. urine).
  The classification also applies to strains that show "intermediate susceptibility" to a more toxic antimicrobial (e.g. aminoglycoside) that cannot be used at a higher dosage. In this situation, the intermediate category serves as a buffer zone between susceptible and resistant.
  As most clinicians are not familiar with the subtle, although clinically important, distinction between intermediate and moderate susceptibility, many laboratories use the designation "intermediate" for reporting purposes.
- *Resistant.* This term implies that the organism is expected not to respond to a given antimicrobial, irrespective of the dosage and of the location of the infection.

In certain situations, for example testing the response of staphylococci to benzylpenicillin, only the categories "susceptible" and "resistant" (corresponding to the production of β-lactamase) are recognized.

The ultimate decision to use a particular antimicrobial, and the dosage to be given, will depend not only on the results of the susceptibility tests, but also on their interpretation by the physician. Other factors, such as pathogenic significance of the microorganism, side-effects and pharmacokinetic properties of the drug, its diffusion in different body sites, and the immune status of the host, will also have to be considered.

Indications for routine susceptibility tests

A susceptibility test may be performed in the clinical laboratory for two main purposes:

- to guide the clinician in selecting the best antimicrobial agent for an individual patient;
- to accumulate epidemiological information on the resistance of microorganisms of public health importance within the community.

Susceptibility tests as a guide for treatment

Susceptibility tests should never be performed on contaminants or commensals belonging to the normal flora, or on other organisms that have no causal relationship to the infectious process. For example, the presence of *Escherichia coli* in the urine in less than significant numbers is not to be regarded as causing infection, and it would be useless and even misleading to perform an antibiogram.

Susceptibility tests should be carried out only on pure cultures of organisms considered to be causing the infectious process. The organisms should also be identified, as not every microorganism isolated from a patient with an infection requires an antibiogram.

Routine susceptibility tests are not indicated in the following situations:

- When the causative organism belongs to a species with predictable susceptibility to specific drugs. This is the case for *Streptococcus pyogenes* and *Neisseria meningitidis*, which are still generally susceptible to benzylpenicillin. (However, there have recently been a few reports of sporadic occurrence of benzylpenicillin-resistant meningococci.) It is also the case for faecal streptococci (enterococci), which, with few exceptions, are susceptible to ampicillin. If resistance of these microorganisms is suspected on clinical grounds, representative strains should be submitted to a competent reference laboratory.
- If the causative organism requires enriched media, e.g. *Haemophilus influenzae* and *Neisseria gonorrhoeae*. Disc-diffusion susceptibility tests may give unreliable results, if the appropriate technique is not strictly followed. The emergence of β-lactamase-producing variants of these species has led to the introduction of special tests, such as the in vitro test for β-lactamase production, described on page 79. It will be the responsibility of the central and regional laboratories to monitor the susceptibility of pneumococci, gonococci, and *Haemophilus.* If problems arise with resistant strains, the peripheral laboratories should be alerted and instructions should be given on appropriate testing methods and on alternative treatment schemes.
- In uncomplicated intestinal infections caused by salmonellae (other than *S. typhi* or *S. paratyphi*). Antimicrobial treatment of such infections is not justified, even with drugs showing in vitro activity. There is now ample evidence that antimicrobial treatment of common salmonella gastroenteritis (and indeed of most types of diarrhoeal disease of unknown etiology) is of no clinical benefit to the patient. Paradoxically, antimicrobials may prolong the excretion and dissemination of salmonellae and may lead to the selection of resistant variants.

Susceptibility tests as an epidemiological tool

Routine susceptibility tests on major pathogens (*S. typhi,* shigellae) are useful as part of a comprehensive programme of surveillance of enteric infections. They are essential for informing the physician of the emergence of resistant strains (chloramphenicol-resistant *S. typhi*, co-trimoxazole-resistant and ampicillin-resistant shigellae) and of the need to modify standard treatment schemes. Although susceptibility testing of non-typhoid salmonellae serotypes causing intestinal infection is not relevant for treating the patient, the appearance of multiresistant strains is a warning to the physician of the overuse and misuse of antimicrobial drugs.

Continued surveillance of the results of routine susceptibility tests is an excellent source of information on the prevalence of resistant staphylococci and Gram-negative bacilli that may be responsible for cross-infections in the hospital. Periodic reporting of the susceptibility pattern of the prevalent strains is an invaluable aid to forming a sound policy on antimicrobial usage in the hospital by restriction and/or rotation of life-saving drugs, such as the aminoglycosides and cefalosporins.

Choice of drugs for routine susceptibility tests in the clinical laboratory

The choice of drugs used in a routine antibiogram is governed by considerations of the antibacterial spectrum of the drugs, their pharmacokinetic properties, toxicity, efficacy and availability, as well as their cost to both the patient and the community. Among the many antimicrobial agents that could be used to treat a patient infected with a given organism, only a limited number of carefully selected drugs should be included in the susceptibility test.

Table 23 indicates the antimicrobials to be tested in various situations. The drugs in the table are divided into two sets. Set 1 includes the drugs that are available in most hospitals and for which routine testing should be carried

Table 23. Basic sets of antimicrobials for routine susceptibility tests[a]

	Staphylococcus	Enterobacteriaceae			*Pseudomonas aeruginosa*
		Intestinal	Urinary	Blood & tissues	
Set 1 First choice	benzylpenicillin oxacillin erythromycin tetracycline chloramphenicol	ampicillin chloramphenicol co-trimoxazole nalidixic acid tetracycline	sulfonamide trimethoprim co-trimoxazole ampicillin nitrofurantoin nalidixic acid tetracycline	ampicillin chloramphenicol co-trimoxazole tetracycline cefalotin gentamicin amoxy-clav[b]	piperacillin gentamicin tobramycin
Set 2 Additional drugs	gentamicin amikacin co-trimoxazole clindamycin nitrofurantoin	norfloxacin	norfloxacin chloramphenicol gentamicin amoxy-clav[b]	cefuroxime ceftriaxone ciprofloxacin piperacillin amikacin	amikacin ciprofloxacin ceftazidine

[a]Notes on the individual antimicrobial agents are given in the text.
[b]Amoxycillin and clavulanic acid (inhibitor of β-lactamase).

out for every strain. Tests for drugs in set 2 are to be performed only at the special request of the physician, or when the causative organism is resistant to the first-choice drugs, or when other reasons (allergy to a drug, or its unavailability) make further testing justified. Many antimicrobials with good clinical activity have been omitted from the table, but it must be emphasized that they are rarely needed in the management of the infected patient. In very rare cases, one or more additional drugs should be included when there is a special reason known to the physician, or when new and better drugs become available. Periodic revision of this table is therefore desirable, and this should be done after appropriate discussions with clinical staff. Many problems arise in practice, because clinicians are not always aware that only one representative of each group of antimicrobial agents is included in routine tests. The result obtained for this particular drug may then be extrapolated to all, or most, of the other members of the group. Difficulties arise in some countries when the physician is familiar only with the commercial brand name of the drug and not with its generic nonproprietary name. A serious effort should be made to inform medical personnel about the international nonproprietary names of pharmaceutical substances, and to encourage their use.[1]

1. The *benzylpenicillin* disc is used to test susceptibility to all β-lactamase-sensitive penicillins (such as oral phenoxymethylpenicillin and pheneticillin). Isolates of staphylococci that fall into the resistant category produce β-lactamase and should be treated with a β-lactamase-resistant penicillin G or with another antimicrobial, such as erythromycin.
2. The *oxacillin* disc is representative of the whole group of β-lactamase-resistant penicillins (including meticillin, nafcillin, cloxacillin, dicloxacillin, and flucloxacillin). There is good clinical evidence that cross-resistance exists between the meticillin and the cefalosporin groups. Therefore, it is useless and misleading to include cefalotin in the antibiogram for staphylococci.
   Resistance to meticillin and related drugs is often of the heterogeneous type, i.e. the majority of cells may be fully susceptible and produce a wide inhibition zone, while the resistant part of the population appears in the form of minute discrete colonies growing within the inhibition zone. This type of resistance is more apparent when the temperature of the incubator is set at 35 °C[2] or when the incubation time is prolonged.
   A serious disadvantage of meticillin, as a representative disc for the β-lactamase-resistant penicillins, is its great lability even under conventional storage conditions. The oxacillin disc is much more resistant to deterioration and is therefore preferred for the standardized diffusion test. The cloxacillin and dicloxacillin discs are not used as they may not indicate the presence of a heteroresistant strain.
3. The results for the *tetracycline* disc may be applied to chlortetracycline, oxytetracycline, and other members of this group. However, most tetracycline-resistant staphylococci remain normally sensitive to minocycline. A disc of minocycline may thus be useful to test multiresistant strains of staphylococci.
4. The result for the *chloramphenicol* disc may be extrapolated to thiamphenicol, a related drug with a comparable antimicrobial spectrum, but without known risk of aplastic anaemia.
5. Only one representative of *sulfonamide* (sulfafurazole) is required in the test.

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[1] *International Nonproprietary Names (INN) for Pharmaceutical Substances.* Cumulative list No. 9. Geneva, World Health Organization, 1996.

[2] Sahm DF et al. Current concepts and approaches to antimicrobial agent susceptibility testing. In: *Cumitech 25*, Washington, DC, American Society for Microbiology, 1988.

6. The *co-trimoxazole* disc contains a combination of trimethoprim and a sulfonamide (sulfamethoxazole). The two components of this synergistic combination have comparable pharmacokinetic properties and generally act as a single drug.
7. *Ampicillin* is the prototype of a group of broad-spectrum penicillins with activity against many Gram-negative bacteria. As it is susceptible to β-lactamase, it should not be used for testing staphylococci. Generally, the susceptibility to ampicillin is also valid for other members of this group: amoxycillin, pivampicillin, talampicillin, etc. (although amoxycillin is twice as active against salmonellae and only half as active against shigellae and *H. influenzae*).
8. Only *cefalotin* needs to be tested routinely as its spectrum is representative of all other first-generation cefalosporins (cefalexin, cefradine, cefaloridine, cefazolin, cefapirin). Where second- and third-generation cefalosporins and related compounds (cefamycins) with an expanded spectrum are available, a separate disc for some of these new drugs may be justified in selected cases (cefoxitin, cefamandole, cefuroxime, cefotaxime, ceftriaxone). Although some cefalosporins can be used to treat severe staphylococcal infections, the susceptibility of the infecting strain can be derived from the result with oxacillin as already mentioned under 2 above.
9. *Erythromycin* is used to test the susceptibility to some other members of the macrolide group (oleandomycin, spiramycin).
10. *Aminoglycosides* form a group of chemically related drugs that includes streptomycin, gentamicin, kanamycin, netilmicin and tobramycin. Their antimicrobial spectra are not always closely enough related to permit assumption of cross-resistance, but against susceptible pathogens these agents have been shown to be equally effective. Numerous studies have compared the nephrotoxicity and ototoxicity of gentamicin, netilmicin and tobramycin, but there is no conclusive evidence that any one of the drugs is less toxic than the others. It is strongly recommended that each laboratory select a single agent for primary susceptibility testing. The other agents should be held in reserve for treatment of patients with infections caused by resistant organisms.
11. *Nitrofurantoin* is limited to use only in the treatment of urinary tract infections, and should not be tested against microorganisms recovered from material other than urine.

Table 24 provides information on zone diameter limits for control strains.

The modified Kirby–Bauer method

The disc-diffusion method, originally described in 1966,[1] is well standardized and has been widely evaluated. Official agencies have recommended it, with minor modifications, as a reference method which could be used as a routine technique in the clinical laboratory.

[1] Bauer AW et al. Antibiotic susceptibility testing by a standardized single disc method. *American journal of clinical pathology*, 1966; **45**:493–496.

Table 24. Zone diameter limits for control strains[a]

Antimicrobial	Disc potency	Diameter of zone of inhibition (mm)		
		S. aureus (ATCC 25923)	*E. coli* (ATCC 25922)	*P. aeruginosa* (ATCC 27853)
amikacin	30 µg	20–26	19–26	18–26
amoxy-clav[b]	20/10 µg	28–36	19–25	—
ampicillin	10 µg	27–35	16–22	—
benzylpenicillin	10 IU	26–37	—	—
cefalotin	30 µg	29–37	15–21	—
cefalozin	30 µg	29–35	23–29	—
ceftazidime	30 µg	16–20	25–32	22–29
cefotaxime	30 µg	25–31	29–35	18–22
ceftriaxone	30 µg	22–28	29–35	17–23
cefuroxime	30 µg	27–35	20–26	—
chloramphenicol	30 µg	19–26	21–27	—
ciprofloxacin	5 µg	22–30	30–40	25–33
clindamycin	2 µg	24–30	—	—
co-trimoxazole	25 µg	24–32	24–32	—
erythromycin	15 µg	22–30	—	—
gentamicin	10 µg	19–27	19–26	16–21
nalidixic acid	30 µg	—	22–28	—
nitrofurantoin	300 µg	18–22	20–25	—
norfloxacin	10 µg	17–28	28–35	22–29
oxacillin	1 µg	18–24	—	—
piperacillin	100 µg	—	24–30	25–33
sulfonamide[c]	300 µg	24–34	15–23	—
tetracycline	30 µg	24–30	18–25	—
tobramycin	10 µg	19–29	18–26	19–25
trimethoprim	5 µg	19–26	21–28	—
vancomycin	30 µg	17–21	—	—

[a]National Committee for Clinical Laboratory Standards. *Performance standards for antimicrobial disc susceptibility tests.* 6th ed. Vol. 21 No 1 (M2-A7 and M7-A5) and 11th informational supplement 2001 (M100-S11).
[b]Amoxycillin and clavulanic acid (inhibitor of β-lactamase).
[c]Sulfisoxazole.

Reagents

Mueller–Hinton agar

1. Mueller–Hinton agar should be prepared from a dehydrated base according to the manufacturer's instructions. The medium should be such that control zone sizes within the published limits are produced (see Table 24). It is important not to overheat the medium.
2. Cool the medium to 45–50 °C and pour into the plates. Allow to set on a level surface, to a depth of approximately 4 mm. A 9-cm plate requires approximately 25 ml of medium.
3. When the agar has solidified, dry the plates for immediate use for 10–30 minutes at 35 °C by placing them in the upright position in the incubator with the lids tilted.
4. Any unused plates may be stored in a plastic bag, which should be sealed and placed in the refrigerator. Plates stored in this way will keep for 2 weeks.

To ensure that the zone diameters are sufficiently reliable for testing susceptibility to sulfonamides and co-trimoxazole, the Mueller–Hinton agar must have low concentrations of the inhibitors thymidine and thymine. Each new lot of Mueller–Hinton agar should therefore be tested with a control strain of *Enterococcus faecalis* (ATCC 29212 or 33186) and a disc of co-trimoxazole. A satisfactory lot of medium will give a distinct inhibition zone of 20 mm or more that is essentially free of hazy growth or fine colonies.

Antimicrobial discs

Any commercially available discs with the proper diameter and potency can be used. Stocks of antimicrobial discs should preferably be kept at –20 °C; the freezer compartment of a home refrigerator is convenient. A small working supply of discs can be kept in the refrigerator for up to 1 month. On removal from the refrigerator, the containers should be left at room temperature for about 1 hour to allow the temperature to equilibrate. This procedure reduces the amount of condensation that occurs when warm air reaches the cold container. If a disc-dispensing apparatus is used, it should have a tight-fitting cover and be stored in the refrigerator. It should also be allowed to warm to room temperature before being opened.

Turbidity standard

Prepare the turbidity standard by pouring 0.6 ml of a 1% (10 g/l) solution of barium chloride dihydrate into a 100-ml graduated cylinder, and filling to 100 ml with 1% (10 ml/l) sulfuric acid. The turbidity standard solution should be placed in a tube identical to the one used for the broth sample. It can be stored in the dark at room temperature for 6 months, provided it is sealed to prevent evaporation.

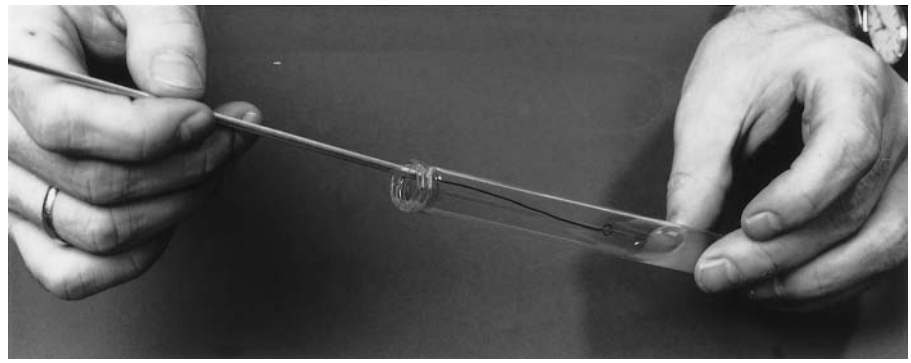
Swabs

A supply of cotton wool swabs on wooden applicator sticks should be prepared. They can be sterilized in tins, culture tubes, or on paper, either in the autoclave or by dry heat.

Procedure

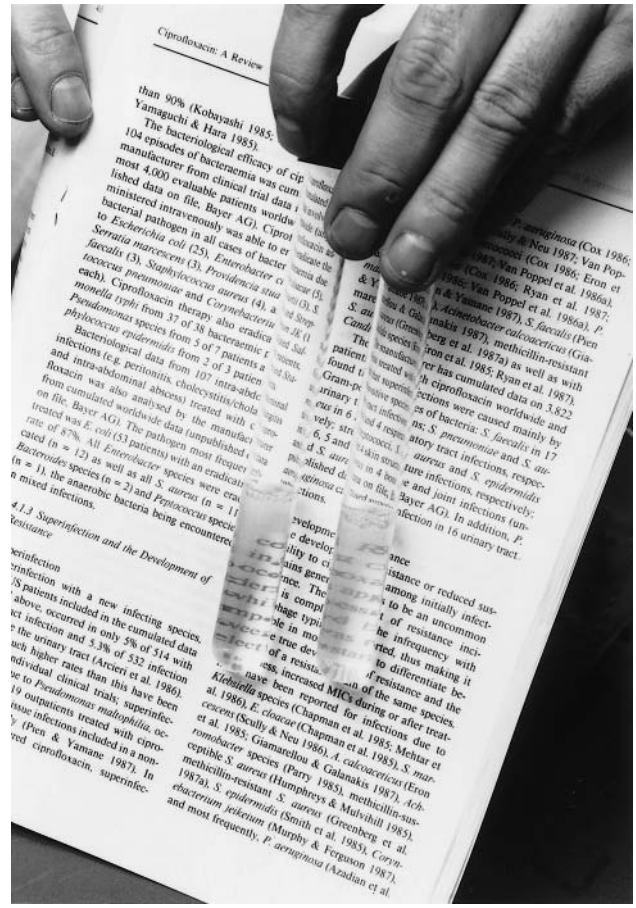
To prepare the inoculum from the primary culture plate, touch with a loop the tops of each of 3–5 colonies, of similar appearance, of the organism to be tested.

Transfer this growth to a tube of saline.



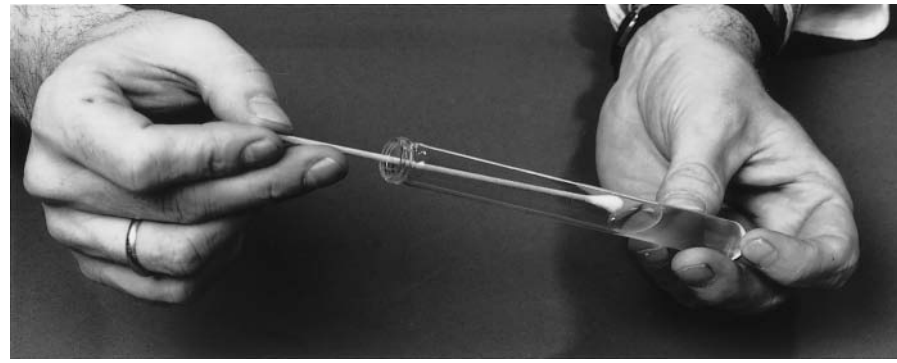
When the inoculum has to be made from a pure culture, a loopful of the confluent growth is similarly suspended in saline.

Compare the tube with the turbidity standard and adjust the density of the test suspension to that of the standard by adding more bacteria or more sterile saline.

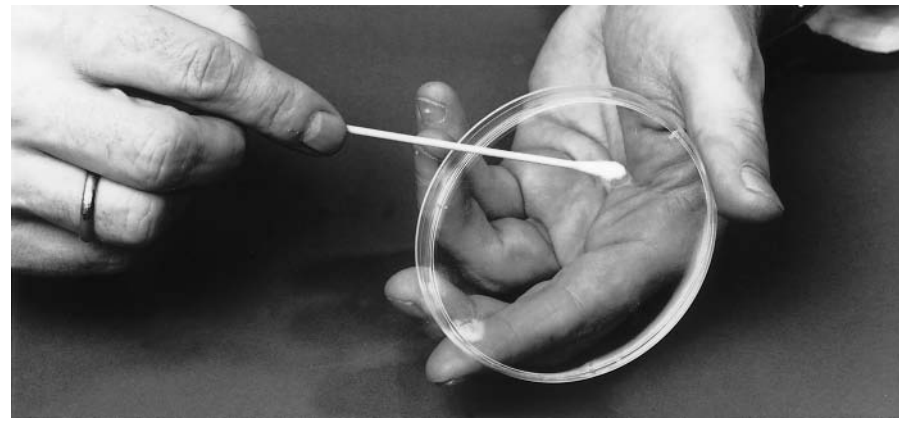


Proper adjustment of the turbidity of the inoculum is essential to ensure that the resulting lawn of growth is confluent or almost confluent.

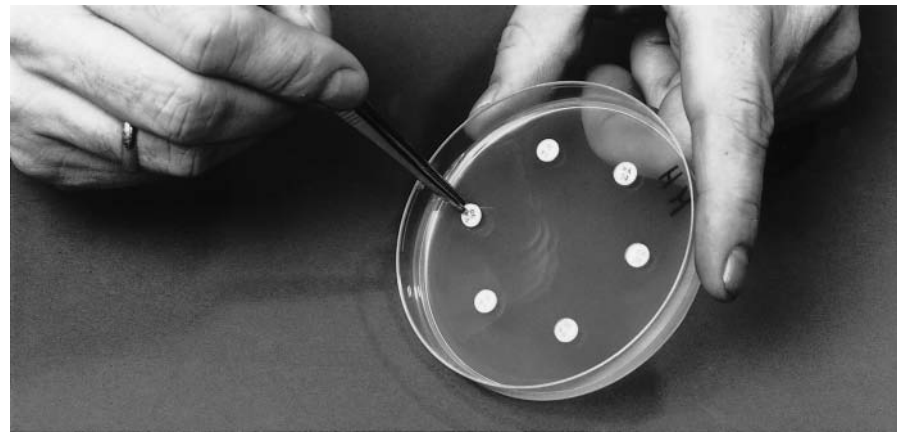
Inoculate the plates by dipping a sterile swab into the inoculum. Remove excess inoculum by pressing and rotating the swab firmly against the side of the tube above the level of the liquid.



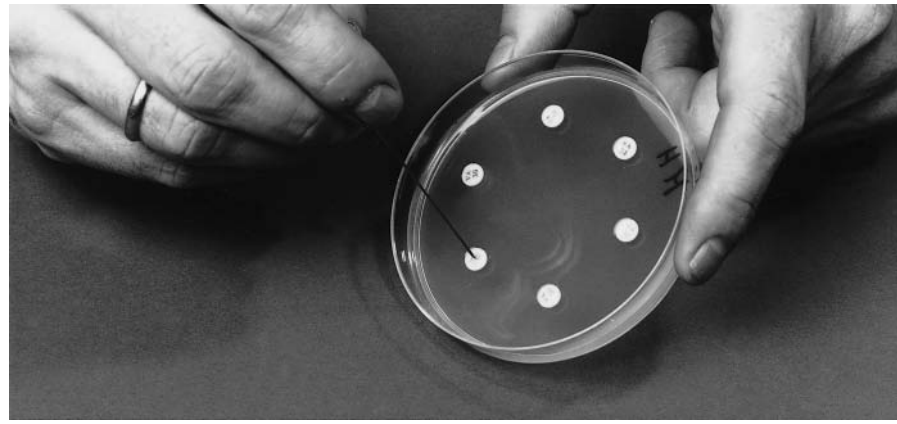
Streak the swab all over the surface of the medium three times, rotating the plate through an angle of 60° after each application. Finally, pass the swab round the edge of the agar surface. Leave the inoculum to dry for a few minutes at room temperature with the lid closed.



The antimicrobial discs may be placed on the inoculated plates using a pair of sterile forceps. It is convenient to use a template (Fig. 15) to place the discs uniformly.



A sterile needle tip may also be used to place the antimicrobial discs on the inoculated plate.



Alternatively, an antimicrobial disc dispenser can be used to apply the discs to the inoculated plate.

A maximum of seven discs can be placed on a 9–10 cm plate. Six discs may be spaced evenly, approximately 15 mm from the edge of the plate, and 1 disc placed in the centre of the plate. Each disc should be gently pressed down to ensure even contact with the medium.

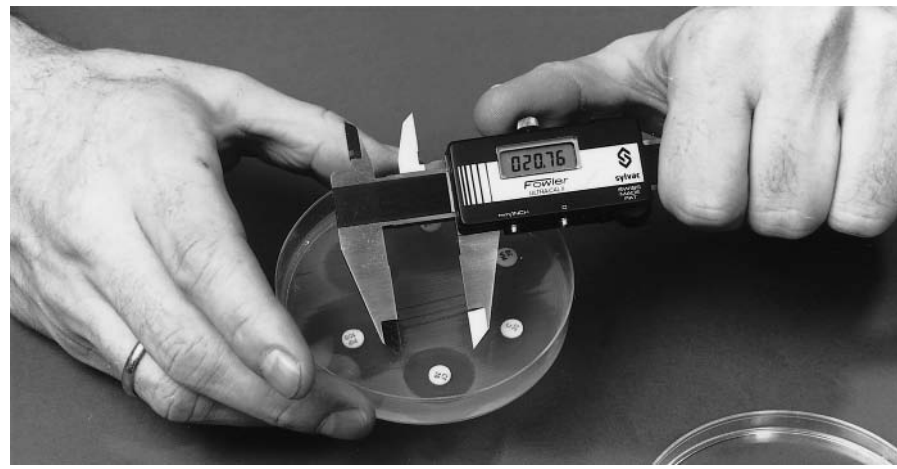
The plates should be placed in an incubator at 35 °C within 30 minutes of preparation. Temperatures above 35 °C invalidate results for oxacillin/meticillin.

Do not incubate in an atmosphere of carbon dioxide.

After overnight incubation, the diameter of each zone (including the diameter of the disc) should be measured and recorded in mm. The results should then be interpreted according to the critical diameters shown in Table 25.

The measurements can be made with a ruler on the under-surface of the plate without opening the lid.

If the medium is opaque, the zone can be measured by means of a pair of calipers.



A template (Fig. 14) may be used to assess the final result of the susceptibility tests.

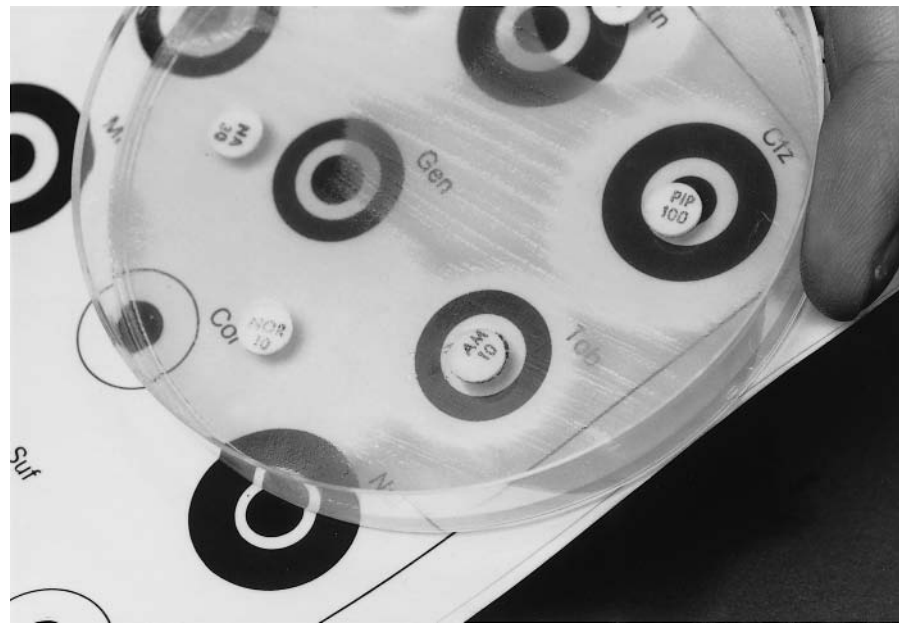


Table 25. Interpretative chart of zone sizes for rapidly growing bacteria using the modified Kirby–Bauer technique[a]

Antimicrobial agent	Diameter of zone of inhibition (mm)			
	Disc potency	Resistant	Intermediate	Susceptible
amikacin	30 μg	<14	15–16	>17
amoxy-clav[b]	20/10 μg	<13	14–17	>18
ampicillin, when testing				
— Enterobacteriaceae	10 μg	<13	14–16	>17
— enterococci	10 μg	<16	—	>17
benzylpenicillin, when testing				
— staphylococci	10 IU	<28	—	>29
— enterococci	10 IU	<14	—	>15
cefalotin	30 μg	<14	15–17	>18
cefalozin	30 μg	<14	15–17	>18
cefotaxime	30 μg	<14	15–22	>23
ceftazidime	30 μg	<14	15–17	>18
ceftriaxone	30 μg	<13	14–20	>21
cefuroxime sodium, cefamandole	30 μg	<14	15–17	>18
chloramphenicol	30 μg	<12	13–17	<18
ciprofloxacin	5 μg	<15	16–20	>21
clindamycin	2 μg	<14	15–20	⩾21
co-trimoxazole	25 μg	<10	11–15	⩾16
erythromycin	15 μg	<13	14–22	>23
gentamicin	10 μg	<12	13–14	>15
nalidixic acid[c]	30 μg	<13	14–18	⩾19
nitrofurantoin[c]	300 μg	<14	15–16	>17
norfloxacin[c]	10 μg	<12	13–16	>17
oxacillin	1 μg	<10	10–12	>13
piperacillin, when testing				
— *P. aeruginosa*	100 μg	<17	—	>18
— other Gram-negative rods	100 μg	<17	18–20	>21
sulfonamide[c,d]	300 μg	<12	13–16	>17
tetracycline	30 μg	<14	15–18	>19
tobramycin	10 μg	<12	13–14	>15
trimethoprim[c]	5 μg	<10	11–15	⩾16
vancomycin, when testing				
— staphylococci	30 μg	—	—	>15
— enterococci	30 μg	<14	15–16	>17

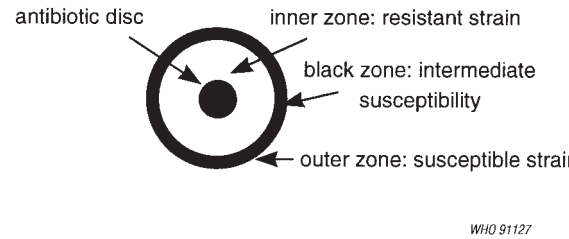
[a]National Committee for Clinical Laboratory Standards. *Performance standards for antimicrobial disc susceptibility tests*, 6th ed., Vol. 21, No. 1 (M2–A7 and M7–A5) Wayne, PA, NCCLS, 1997; and *11th informational supplement* 2001 (M100–S11).

[b]Amoxycillin and clavulanic acid (inhibitor of β-lactamase).

[c]Only applicable for testing isolates from urinary tract infection and some enteric pathogens.

[d]Sulfisoxazol.

Fig. 14. Template for the determination of susceptibility



The end-point of inhibition is judged by the naked eye at the edge where the growth starts, but there are three exceptions:

- With sulfonamides and co-trimoxazole, slight growth occurs within the inhibition zone; such growth should be ignored.
- When β-lactamase-producing staphylococci are tested against benzyl penicillin, zones of inhibition are produced with a heaped-up, clearly defined edge; these are readily recognizable when compared with the sensitive control, and, regardless of the size of the zone of inhibition, they should be reported as resistant.
- Certain *Proteus* species may swarm into the area of inhibition around some antimicrobials, but the zone of inhibition is usually clearly outlined and the thin layer of swarming growth should be ignored.

Interpretation of the zone sizes

Using a template. When the zone sizes are compared with a template, a separate template must be prepared for each individual antimicrobial agent (see Fig. 14). The result—susceptible, resistant, or intermediate—can be read at once: "susceptible", when the zone edge is outside the black circle; "resistant", when there is no zone, or when it lies within the white circle; and "intermediate", when the edge of the zone of inhibition lies on the black circle.

Using a ruler. When the zone sizes are measured in mm, the results should be interpreted according to the critical diameters given in Table 25.

Direct versus indirect susceptibility tests

In the standardized method outlined above, the inoculum is prepared from colonies on a primary culture plate or from a pure culture. This is called an "indirect susceptibility test". In certain cases, where a rapid answer is important, the standardized inoculum may be replaced by the pathological specimen itself, e.g. urine, a positive blood culture, or a swab of pus. For specimens of urine, a microscopical examination of the sediment should first be made in order to see if there is evidence of infection, i.e. the presence of pus cells and/or organisms. The urine may then be used as the inoculum in the standard test. Likewise, susceptibility tests may be performed on incubated blood cultures showing evidence of bacterial growth, or a swab of pus may be used as a direct inoculum, when a Gram-stained smear shows the presence of large numbers of a single type of organism. This is called a "direct susceptibility test"; its advantage over the indirect test is that a result is obtained 24 hours earlier. The main disadvantage is that the inoculum cannot be properly controlled. When the susceptibility plate shows too light or too heavy growth, or when the culture is a mixture, the results should be interpreted with caution, and the test repeated on pure cultures.

Technical factors influencing the size of the zone in the disc-diffusion method

Inoculum density

If the inoculum is too light, the inhibition zones will be larger even though the sensitivity of the organism is unchanged. Relatively resistant strains may then be reported as susceptible. Conversely, if the inoculum is too heavy, the zone size will be reduced and susceptible strains may be reported as resistant. Usually optimal results are obtained with an inoculum size that produces near confluent growth.

Timing of disc application

If the plates, after being seeded with the test strain, are left at room temperature for periods longer than the standard time, multiplication of the inoculum may take place before the discs are applied. This causes a reduction in the zone diameter and may result in a susceptible strain being reported as resistant.

Temperature of incubation

Susceptibility tests are normally incubated at 35 °C for optimal growth. If the temperature is lowered, the time required for effective growth is extended and larger zones result. When a heterogeneous resistant strain of *Staphylococcus aureus* is being tested against meticillin (oxacillin), the resistant portion of the population can be detected at 35 °C. At higher temperatures the entire culture appears to be susceptible. At 35 °C or lower temperatures, resistant colonies develop within the zone of inhibition. These resistant colonies can be seen more easily if the plate is left for several hours at room temperature before the result is read. Such colonies should always be identified to check whether they are contaminants.

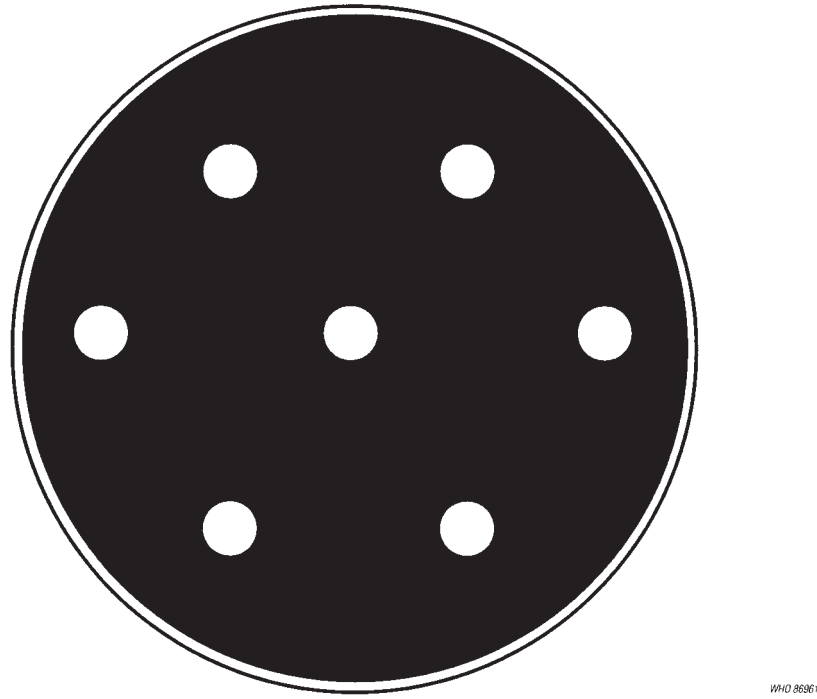
Incubation time

Most techniques adopt an incubation period of between 16 and 18 hours. In emergencies, however, a provisional report may be made after 6 hours. This is not routinely recommended and the result should always be confirmed after the conventional incubation time.

Size of plate, depth of agar medium, and spacing of the antimicrobial discs

Susceptibility tests are usually carried out with 9–10 cm plates and no more than 6 or 7 antimicrobial discs on each plate. If larger numbers of antimicrobials have to be tested, two plates, or one 14-cm diameter plate, is to be preferred. Excessively large inhibition zones may be formed on very thin media; the converse is true for thick media. Minor changes in the depth of the agar layer have negligible effect. Proper spacing of the discs is essential to avoid overlapping of the inhibition zones or deformation near the edge of the plates (see Fig. 15).

Fig. 15. Template for uniform placement of susceptibility discs on plates of 90 mm diameter



Potency of the antimicrobial discs

The diameter of the inhibition zone is related to the amount of drug in the disc. If the potency of the drug is reduced owing to deterioration during storage, the inhibition zone will show a corresponding reduction in size.

Composition of the medium

The medium influences the size of the zone by its effect on the rate of growth of the organism, the rate of diffusion of the antimicrobial, and the activity of the agent. It is essential to use the medium appropriate to the particular method.

The many factors influencing the zone diameters that may be obtained for the same test organism clearly demonstrate the need for standardization of disc-diffusion methods. Only if the conditions laid down in a particular method are closely followed can valid results be obtained. Alteration of any of the factors affecting the test can result in grossly misleading reports for the clinician.

The precision and accuracy of the method should be monitored by establishing the quality control programme described below. Variations can then be immediately investigated and corrective action taken to eliminate them.

Quality control

The need for quality control in the susceptibility test

The final result of a disc-diffusion test is influenced by a large number of variables. Some of the factors, such as the inoculum density and the incubation temperature, are easy to control, but a laboratory rarely knows the exact composition of a commercial medium or the batch-to-batch variations in its quality, and it cannot take for granted the antimicrobial content of the discs. The results of the test must therefore be monitored constantly by a quality control programme, which should be considered part of the procedure itself.

The precision and accuracy of the test are controlled by the parallel use of a set of control strains, with known susceptibility to the antimicrobial agents. These quality control strains are tested using exactly the same procedure as for the test organisms. The zone sizes shown by the control organisms should fall within the range of diameters given in Table 24. When results regularly fall outside this range, they should be regarded as evidence that a technical error has been introduced into the test, or that the reagents are at fault. Each reagent and each step in the test should then be investigated until the cause of the error has been found and eliminated.

Standard procedure for quality control

The quality control programme should test standard reference strains of bacteria in parallel with the clinical cultures. These tests should preferably be run every week, or with every fifth batch of tests, and, in addition, every time a new batch of Mueller–Hinton agar or a new batch of discs is used.

Standard strains for quality control

Staphylococcus aureus (ATCC 25923)
Escherichia coli (ATCC 25922)
Pseudomonas aeruginosa (ATCC 27853)

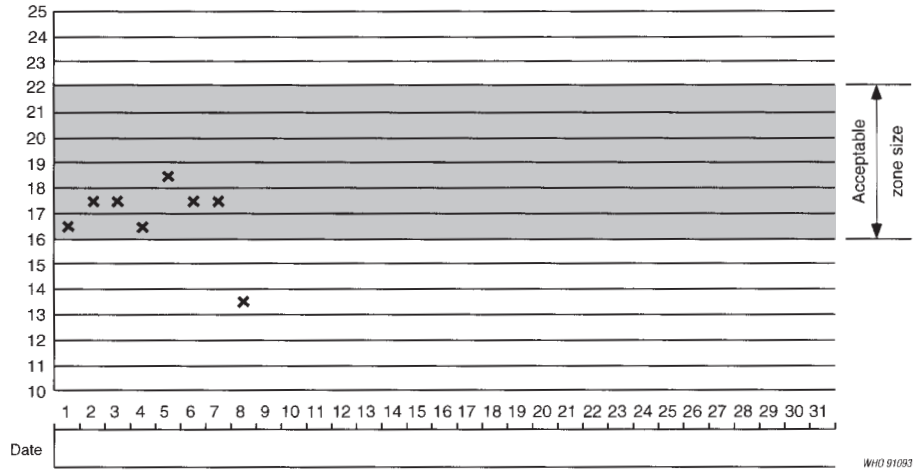
These cultures can be obtained from national culture collections. They are commercially available in the form of pellets of desiccated pure cultures.

Cultures for day-to-day use should be grown on slants of nutrient agar (tryptic soy agar is convenient) and stored in the refrigerator. They should be subcultured on to fresh slants every 2 weeks.

Preparing the inoculum

The cultures may be inoculated into any type of broth, and incubated until the broth is turbid. Each broth should be streaked onto an agar plate and incubated overnight. Single colonies should then be picked off and submitted to susceptibility tests as described on pages 111–115.

Fig. 16. Quality control chart for antimicrobial susceptibility testing



Placing antimicrobial discs

After the inoculum has been streaked on to the plates, as described on page 113, the appropriate discs should be applied. The discs to be selected for each control strain are listed in Table 24.

Reading the plates

After 16–18 hours incubation, the diameters of the inhibition zones should be measured with a ruler and recorded, together with the date of the test, on a special quality control chart. This chart should display data for each disc–strain combination. The chart is labelled in millimetres, with an indication of the range of acceptable zone sizes. An example of such a chart is shown in Fig. 16. When the results consistently fall outside the acceptable limits, action should be taken to improve the quality of the test.

Grossly aberrant results, which cannot be explained by technical errors in the procedure, may indicate contamination or sudden changes in the susceptibility or growth characteristics of the control strain. If this occurs, a fresh stock-strain should be obtained from a reliable source.

Serological tests

Introduction

Serological tests, unlike culture and microscopic investigations, provide only indirect evidence of infection by detecting bacterial antigens, or antibodies produced in response to them, in clinical material. Such tests are now widely used in microbiology because of their high specificity and sensitivity.

After an initial infection with pathogenic microorganisms, most patients will produce both IgM and IgG antibodies. After a few weeks, the cells producing IgM antibodies switch to producing IgG antibodies, and subsequently only IgG antibodies will be present in the patient's serum. A second infection with the same pathogens will elicit only an IgG response. Because the antibody-producing cells have retained the memory of the pathogen, the response will be more rapid and usually stronger than the initial response; this is an anamnestic response.

The antibody level is usually designated by a "titre". The diagnostic titre to be reported is the reciprocal value of the highest dilution of the patient's serum in which antibodies are still detectable. For example, if antibodies are detectable in serum diluted 1:1024 but not in further dilutions, the titre of the serum is 1024. Serum collected during the acute phase of the infection, when the disease is first suspected, is called the acute serum; serum drawn during convalescence, which is usually 2 weeks later, is called convalescent serum.

Reaction to an antigen will occur regardless of the stage of infection, although this reaction will vary. The presence of IgG antibodies in a single serum sample may indicate past exposure to the agent and therefore cannot be used to diagnose a current infection. An antigen may also stimulate production of antibodies that cross-react with other antigens. As these antibodies are nonspecific, tests involving only single serum samples may give rise to misinterpretation of results. For the majority of serological tests, both acute and convalescent sera should be tested, preferably together in the same test-run, to counteract variables inherent in the test procedure. An increase in the antibody titre of two doubling dilutions (e.g. from a dilution of 1:8 to a dilution 1:32) is usually considered to be diagnostic of current infection. This is called a fourfold rise in titre. Testing a single serum sample may be useful only in special cases, such as for diagnosis of *Mycoplasma pneumoniae* infection, where high titres may indicate a recent infection, or when the test demonstrates the presence of IgM antibodies and the result may be taken as evidence of a current infection. The type of antigen–antibody reaction depends on the physical state of the antigen.

Quality control measures

The reliability and consistency of serological test results are entirely dependent on the quality control measures undertaken before, during and after each test. These quality control measures are extremely important as a false-positive or false-negative result might give rise to a medical decision or action that could harm the patient. Many variables may affect the quality of serological testing, including the experience of the laboratory personnel, the quality of the kits and equipment, the condition of the specimens, the controls used in test-runs, and the interpretation and reporting of results. After the test

has been completed, discard used materials into a container filled with disinfectant, and wash hands and bench surfaces with disinfectant.

Equipment

The equipment in a serology laboratory includes water-baths, incubators, refrigerators, freezers, pH meters, balances, centrifuges, microscopes, and rotators. Monitoring and routine maintenance of equipment is a vital part of the quality assurance programme in the serology laboratory. A routine maintenance service should be established with periodic inspection of all equipment for minor adjustments. Records showing dates of inspection, maintenance, and repair should be maintained for each piece of equipment.

The water-bath should be kept free of all foreign material and drained and cleaned monthly. The temperatures of the water-bath should be strictly controlled and should not vary by more than ±1 °C; it should be checked and recorded daily and during use. A cover should be provided to prevent cooling at the surface of the water. Mixtures of antigen and antibody should not be incubated until the desired temperature has been maintained for at least one hour. The water level should be the same as that of the fluid in the tubes or flasks that are placed in the water-bath.

Microscopes are essential for the Venereal Disease Research Laboratory (VDRL) test and the fluorescent treponemal antigen absorption (FTA-Abs) test, and must be maintained in the best possible working order. After use, the eyepieces, objectives, and condenser of the microscope should be wiped clean of all oil and debris with a non-abrasive tissue. When not in use, the microscope should be kept covered and protected against moisture. The intensity of the mercury lamp of the fluorescence microscope should be checked repeatedly with a light meter.

The mechanical rotator for the VDRL and the rapid plasma reagin (RPR) tests should be checked for speed during use, and any change in speed that may adversely affect the agglutination reaction should be corrected. The mechanical rotator should be lubricated on a regular basis.

Materials

Glassware and needles used in serological tests should meet the specifications recommended in those tests.

All chipped or scratched glassware, plates and slides should be discarded. The use of dirty or improperly cleaned glassware that may have residues of organic material on it is a major cause of misleading results. The instructions for washing glassware should be clearly written out and displayed in the washing area. The basic steps should include pre-rinsing, washing with an appropriate laboratory detergent, rinsing with tap water followed by distilled water, drying, and checking to ensure that all detergent has been removed.

All pipettes should be soaked upright in a detergent solution so that the solution fills the inside of the pipette. Pipettes should then be rinsed for at least 30 minutes under running water, followed by a rinse in distilled water. Glass plates used for the VDRL test must be cleaned thoroughly until all detergent and oil residues are removed. Prolonged soaking in detergent solutions should be avoided as this may weaken and flake the ceramic rings on the

plates. Glass plates with paraffin rings should be cleaned using an appropriate organic solvent (e.g. petrol ether).

For the RPR and VDRL tests, calibrated needles are used for dispensing antigen and diluent. The RPR test kit includes a dispensing needle, and these needles should be checked before use. Check the needle by determining the number of drops/ml: a 20-gauge needle should deliver 90 drops/ml. RPR needles that are not working properly should be discarded. Wash the needles with distilled water after use. To prepare the two needles for the VDRL test, break the points with a pair of pliers. Check the needles by determining the number of drops/ml: an 18-gauge needle must deliver 60 drops/ml, and a 21- or 22-gauge needle deliver 100 drops/ml. Any needle in the VDRL test kit that does not deliver the exact number of drops should be discarded, or adjusted by either pressing or reaming the end of the needle. Wash the needles with water, 70% ethanol and acetone after use.

Reagents

Chemicals used in serology must be of reagent quality and must meet the specifications of the particular procedure. They should be stored in accordance with the manufacturers' instructions.

High-quality distilled water with a pH of 7.0 should be used for the preparation of reagents. Distilled water should be stored in a heat-resistant glass or plastic bottle with a tightly fitting lid and be properly labelled and dated.

Saline is used either as saline or as a buffered solution, such as phosphate-buffered saline. In humid climates, sodium chloride should be dried in a hot-air oven for 30 minutes at 160–180 °C to remove any moisture. The salt should be dissolved in distilled or demineralized water and stored in a heat-resistant glass or plastic bottle with a tightly fitting lid and be properly labelled and dated. When buffered saline is prepared, the pH must be determined before use.

Sera that contain particulate debris should be centrifuged at 3000*g* for 10 minutes, and the supernatant used for testing. Haemolysed or contaminated sera should be discarded. If inactivated serum is required for a test, heat the serum at 56 °C for 30 minutes. If the serum has not been used 4 hours after the initial inactivation, it should be re-inactivated by heating at 56 °C for 10 minutes before testing. Bring all sera to room temperature before testing.

A detailed description of some of the serological procedures routinely performed in many medical laboratories will be discussed in this chapter. These include serological tests for the investigation of syphilis, the Wright test for the diagnosis of brucellosis, and the antistreptolysin O test for the diagnosis of post-streptococcal disease.

Each description refers to the use of a commercial test kit. As these test kits are available from a number of manufacturers, the user should carefully read the detailed instructions contained in each package insert.

Serological reactions

Flocculation or precipitin reactions

In flocculation tests, the antigen is in solution and the interaction with the antibody will result in the formation of a precipitate, which can be observed either under the microscope or with the naked eye. When the reagents are mixed, the initial combination of antigen and antibody occurs almost immediately. However, subsequent formation of larger visible clumps requires an hour or more and is temperature-dependent. The reaction is fastest in the zone of equivalence, where the antigen–antibody ratio is optimal. The tube with the quickest formation of a precipitate is a good indication of equivalence. The most widely used flocculation tests are the VDRL and the RPR test. Both are used for the diagnosis of syphilis (caused by *Treponema pallidum*), and for other treponemal infections.

Flocculation tests provide qualitative evidence of an antigen–antibody reaction but do not indicate whether one or more types of antigen–antibody reactions are involved. If, however, the reactions are investigated using a semisolid gel, the different antigens and antibodies are likely to diffuse at different migration rates, and it may be possible to distinguish different reactions.

Agglutination reactions

In agglutination tests, the reagent, which may be an antigen or an antibody, is fixed or absorbed to a micro-particle. A variety of particles can be used as carriers of the reagent, e.g. latex particles, gelatin particles, microbeads, bacteria or red blood cells. This technique is also called passive agglutination. When red blood cells are used as carriers the tests are called coagglutination tests. When mixed with a specific antiserum, the cells or particles form a lattice network that results in clumping and leaves a clear supernatant. If an antiserum of known specificity is used, the test for the identification of unknown microorganisms or their antigens can be applied. This test may be performed on a slide, and the resulting agglutination read macroscopically or under the low-power objective of microscope. The agglutination reaction is also used to estimate the titre of antibacterial agglutinins in the serum of patients with unknown diseases. A rise in titre during an illness strongly suggests a causal relationship.

Agglutination is accelerated at higher temperatures (35–56 °C) and by movement (e.g. shaking, stirring or centrifuging), which increases the contact between antigen and antibody. The agglutination process requires the presence of salts. A potentially serious problem with agglutination tests is prozone reaction: if too much antibody is present, the lattice will not form and agglutination will be inhibited. A prozone reaction gives the impression that antibody is absent; this error, however, can be avoided by testing serial dilutions of the serum.

There is a commonly used agglutination test that uses *Staphylococcus aureus*, which contains a protein, Protein A, on its surface. This protein binds to the Fc fragment of IgG antibodies. IgG antibody-coated staphylococci produce visible agglutination in the presence of a specific antigen. The test is mainly used to identify organisms cultured from clinical specimens or to detect bacterial antigens in body fluids of infected patients (cerebrospinal fluid in the case of meningitis).

Agglutination tests are used for the diagnosis of Epstein-Barr virus (infectious mononucleosis), rotavirus, rubella, and for the detection of bacterial antigens (*Haemophilus* and *Streptococcus* A and B, among others).

Fluorescent antibody tests

In immunofluorescence tests, the immunoreagent (antigen or antibody) is attached to a fluorescent dye, such as fluorescein or rhodamine, and the reaction between the antigen and the antibody is detected by fluorescence microscopy. In the direct antigen-detection test, fluorescein-conjugated antibodies are used to reveal the presence of a specific antigen. The test is a valuable aid in the rapid identification of *Chlamydia trachomatis, C. psittaci, Rickettsia* spp., *Streptococcus pyogenes, Bordetella pertussis, Corynebacterium diphtheriae, Legionella pneumophila*, and other organisms in clinical specimens.

In the indirect fluorescent antibody (IFA) test, serial dilutions of a patient's serum are allowed to react with the specific antigen, and antihuman IgG or IgM antibodies conjugated to fluorescein are added to make the reaction visible. For example, in the serodiagnosis of syphilis, *Treponema pallidum* antigen is fixed to a slide, overlaid with the patient's serum, and then washed. Fluorescein-labelled antihuman immunoglobulin is then placed on the preparation, which is washed and examined by fluorescence microscopy. If the patient's serum contains specific antibodies to *Treponema pallidum*, brightly fluorescent spirochaetes are seen. If the spirochaetes do not fluoresce, no specific antitreponemal antibodies are present in the patient's serum. The IFA test can also be used to identify other bacteria, including mycobacteria, and this procedure is often more sensitive than the direct immunofluorescence test, because more fluorescein-labelled antibodies will attach to each antigen site.

Serological tests for syphilis

The serological tests for the diagnosis of syphilis include non-treponemal and treponemal tests. The non-treponemal tests are the VDRL and the RPR tests. The antigens used in these tests are prepared from non-treponemal antigens, such as cardiolipin–lecithin, and they detect the antibody-like substance *reagin*, which is present in the sera of many patients with syphilis, and may occasionally be detected in the sera of patients with other acute and chronic diseases. These tests are practical, inexpensive, and reproducible, although they are not absolutely specific. They may confirm the diagnosis of early or late symptomatic syphilis or provide diagnostic evidence of latent syphilis. This test is superior to the treponemal tests as a follow-up investigation after treatment. Moreover, the VDRL test is an effective tool in epidemiological investigations of syphilis and other treponemal diseases.

The treponemal tests use *Treponema pallidum* antigens to detect specific antibodies that have developed in serum in response to syphilis infection. The procedures are used to verify the specificity of positive reactions in non-treponemal tests. The fluorescent treponemal antibody absorption test (FTA-Abs) and the *Treponema pallidum* haemagglutination test (TPHA) are highly specific and sensitive but they cannot differentiate between active and past syphilis infections; neither can they be used for evaluating therapeutic response.

VDRL test

The VDRL test uses cardiolipin–lecithin-coated cholesterol particles. Inactivated serum or cerebrospinal fluid and VDRL antigen emulsion are mixed using a rotating machine for a prescribed period of time. The VDRL particles will flocculate if reagin is present in the serum or cerebrospinal fluid.

The VDRL test is strongly reactive for early syphilis infection. After effective treatment the titre falls gradually and usually becomes non-reactive within 1–2 years. In the late phase of the disease the serum may remain reactive at a low titre (e.g. 1:8 or less) for many years, even after effective treatment. Reactivity may cease spontaneously in about 20–30% of untreated patients during the latent phase of the disease, and even more often during the late phase.

False-positive results may be observed because of the similarity of the VDRL antigen with normal host tissue. Although false-positive reactions can sometimes be observed in the serum of healthy persons, they are often associated with a specific disease or following vaccination. Acute false-positive reactions often have low titres (1:8 or less) and are mainly seen in persons with viral or bacterial infections (atypical pneumonia, psittacosis, infectious mononucleosis and infectious hepatitis), during pregnancy or after recent vaccination. Prolonged false-positive reactions usually have high titres that are caused by autoantibodies (rheumatoid factors) in patients with lepromatous leprosy, tuberculosis, immune disorders (e.g. lupus erythematosus, collagenosis, rheumatic diseases, Sjögren syndrome, dysgammaglobulinaemia) and occasionally malaria, or in those who are dependent on heroin. Reactive or weakly reactive VDRL test results should not be considered conclusive evidence of syphilis in a patient and a non-reactive VDRL test by itself does not rule out the diagnosis of syphilis. Any test sample giving a reactive or weakly reactive result in the absence of clinical evidence of syphilis should therefore be subjected to a treponemal test such as FTA-Abs or TPHA.

Materials and reagents provided in the VDRL test kit

Buffered saline solution
Control sera set (non-reactive, weakly reactive and reactive)
VDRL antigen

Additional materials and reagents required for the VDRL test

Absolute alcohol and acetone
Agglutination slide, approximately 5 × 7.5 cm with wells 16 mm in diameter and 1.75 mm deep, for cerebrospinal fluid tests
Aliquot vials
Distilled or deionized water
Glass plate with 12 paraffin or ceramic rings approximately 14 mm in diameter for serum tests
Humidity cover
Hypodermic needles without bevels: 18-gauge for serum tests, and 21- or 22-gauge for cerebrospinal fluid tests
Interval timer
Light microscope with ×10 ocular and ×10 objective
Mechanical rotator, circumscribing a circle 2 cm in diameter, at a speed of 180 rev/min, on a horizontal plane, with an automatic timer
pH meter
Serological pipettes: 5.0 ml, 1.0 ml and 0.2 ml
Sterile saline solutions (0.85% and 10%)
Syringe, Luer-type, 1 or 2 ml

VDRL antigen emulsion bottles, 30-ml, round, glass-stoppered, narrow-mouth, approximately 35 mm in diameter with flat inner bottom surface
Water-bath (56 °C)

Rehydration of VDRL antigen and control sera

The VDRL antigen is an alcoholic solution of lipids (cardiolipin and lecithin) and cholesterol. These substances are not soluble in water. VDRL antigen is unstable and a fresh suspension must be prepared on the day of use. Pour the contents of the antigen ampoule into the storage vial. Ensure that the vial is tightly capped and store in the dark at 15–30 °C. Withdraw antigen as required.

After the bottle of buffered saline has been opened, it should be stored in the refrigerator. Discard if turbidity appears.

Rehydrate the control sera with 3 ml of distilled or deionized water. Divide the rehydrated sera that are surplus to the day's use into suitable aliquot portions (one day's use) and store at –20 °C for up to one month. Do not thaw and refreeze. Store the sera to be used for the day in the refrigerator at 2–8 °C.

Preparation of VDRL antigen emulsion

1. Allow the antigen and buffered saline to reach room temperature. Check the pH of the buffered saline and discard if outside the range of pH 6.0 ± 0.1.
2. Pipette 0.4 ml of buffered saline into an antigen emulsion bottle and gently tilt the bottle so that the buffered saline covers the bottom the bottle.
3. Measure 0.5 ml of antigen solution using a 1-ml pipette graduated to the tip, and add the antigen as follows:
   - Keep the pipette in the upper one third of the bottle. Do not let it touch the saline.
   - While rotating the bottle manually in a circle approximately 5 cm in diameter, add the antigen drop by drop to the buffered saline.
   - Allow approximately 6 seconds to add the antigen, then discharge the remaining antigen in the pipette into the bottle.
   - Continue rotation of the bottle for 10 seconds.
4. Add 4.1 ml of buffered saline to the bottle, allowing it to flow down the side of the bottle.
5. Place the glass stopper in the bottle and shake the bottle up and down approximately 30 times in 10 seconds.
6. Let the antigen emulsion stand for at least 10 minutes before using. Swirl gently prior to use. The antigen emulsion may be used for the next 8 hours.
7. If cerebrospinal fluid is to be tested, dilute the antigen emulsion a further 1:2 with an equal volume of 10% saline solution. Shake the bottle gently for 10 seconds and allow to stand for a minimum of 5 minutes and a maximum of 2 hours before using.

VDRL qualitative test

1. Using a 1.0 ml pipette, mix the inactivated serum several times then add 0.05 ml to the first well of the VDRL glass plate.
2. Spread the serum with a circular motion of the pipette tip so that it covers the entire inner surface of the paraffin or ceramic well. Use only clean plates that allow the serum to cover evenly the entire surface within the paraffin or ceramic well.

3. Take a syringe with an 18-gauge needle and, holding it vertically, carefully add 1 drop of antigen (1/60 ml) to the serum. Do not allow the needle to touch the serum.
4. Place the plates on the mechanical rotator under a humidity cover and rotate for 4 minutes. If a mechanical rotator is not available rotate the card by hand with a steady circular motion for 4 minutes.
5. Examine the plate immediately after rotation using a microscope with a ×10 ocular and a ×10 objective.
6. Read the reactions as follows:

Medium and large clumps	R	Reactive
Small clumps	W	Weakly reactive
No clumping or very slight roughness	N	Non-reactive

Serum that produces weakly reactive or rough non-reactive results should be retested with the semi-quantitative test as prozone reactions are occasionally encountered.

VDRL semi-quantitative test

1. Prepare a two-fold serial dilution of inactivated serum in 0.85% saline (1:2, 1:4, 1:8, 1:16, 1:32).
2. Test each serum dilution using the qualitative test procedure.
3. Report the results in terms of the highest serum dilution that produces a reactive (not weakly reactive) result in accordance with the following examples:

Dilution						Report
Undiluted serum	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	
W	N	N	N	N	N	Weakly reactive, undiluted
R	W	N	N	N	N	Reactive, undiluted
R	R	W	N	N	N	Reactive, 1:2 dilution
R	R	R	W	N	N	Reactive, 1:4 dilution
W	W	R	R	W	N	Reactive, 1:8 dilution
N (rough)	W	R	R	R	N	Reactive, 1:16 dilution

W: weakly reactive; R: reactive; N: non-reactive.

4. If reactive results are obtained up to dilution 1:32, prepare further twofold serial dilutions in 0.85% saline (1:64, 1:128 and 1:256) and retest using the qualitative test procedure.

RPR test

The antigen suspension in the RPR test contains charcoal particles to allow for macroscopically visible flocculation. The main differences between the RPR test and the VDRL test are that it uses a stabilized antigen, cards instead of plates, and serum as well as plasma, and the serum does not need to be heated. As only a small amount of sample is required, plasma or serum from capillary blood can also be used. The RPR test cannot be used to test cerebrospinal fluid.

In the RPR test the antigen is ready for immediate use. It requires no prior preparation or dilution. Unopened antigen reagent has a shelf-life of one year; storage in a refrigerator is recommended. Once opened, the antigen reagent maintains its reactivity for 3 months when stored in the refrigerator in its plastic dispenser. The RPR test is slightly more sensitive than the VDRL test and it is easier and quicker to perform. False-positive reactions occur slightly more often with the RPR test than with the VDRL test. Some commercial kits require a mechanical rotator for mixing the reagents, whereas others can be rotated manually.

Materials and reagents provided in the RPR test kit

Antigen delivery needle to deliver 60 drops/ml of the antigen suspension
Control sera, positive and negative
Disposable droppers to deliver 50 µl of serum or plasma
Plastic-coated RPR test cards, each with two rows of five wells
Prepared RPR antigen suspension
Stirrers

Additional materials and reagents required for the RPR test

Disposable dropper
Grease pencil
Humidity cover
Mechanical rotator, circumscribing a circle 2 cm in diameter, at a speed of 180 rev/min, on a horizontal plane, with an automatic timer
Sterile saline (0.85%)

RPR qualitative test

1. Remove the reagent kit from the refrigerator and allow the reagents to warm to room temperature.
2. Reconstitute the control serum by adding the recommended volume of distilled water.
3. Label each well on the RPR card with the laboratory number of a sample to be tested, including wells for positive, weakly positive and negative control sera.
4. Use the disposable dropper to add 50 µl of unheated serum or plasma to the corresponding well. Use a new dropper for each sample.
5. Gently shake the antigen suspension and add one free-falling drop to each well using the antigen delivery needle provided. Carefully mix the antigen suspension and serum. Use a new stirrer for each sample. Spread to cover the area of the well.
6. Place the card on the mechanical rotator under a humidity cover and rotate for 8 minutes. If a mechanical rotator is not available rotate the card by hand with a steady circular motion for 2 minutes, then place it in a moist chamber containing wet tissue or filter-paper for 6 minutes. Remove the card and rotate briefly to obtain the final reading. Take care to avoid cross-contamination of samples.
7. Remove the card from the rotator and examine it macroscopically in a good light. The positive control serum should show clearly visible agglutination. The negative control serum should show no agglutination. A brief rotation and tilting of the card by hand can help to differentiate weakly reactive from non-reactive samples.
8. Record the test results:
   - Small to large flocculated clumps: reactive
   - Even turbidity of the particle suspension: non-reactive
9. Prepare serial dilutions of any reactive sera to estimate the antibody titre.

RPR semi-quantitative test

1. Remove the reagent kit from the refrigerator and allow the reagents to warm to room temperature.
2. Label a row of 5 wells on the RPR card with the laboratory number for each sample to be tested.
3. Use the disposable dropper to add 1 drop of saline (0.85%) to each well. Do not spread.
4. Use a new dropper to add 1 drop of the serum sample to the first well. Mix by drawing up and down the dropper 5–6 times (avoid bubble formation).
5. Transfer 50 µl of the mixed sample (1:2 dilution) to the next well. Mix. Repeat the procedure up to the 5th well (1:32 dilution). Discard 50 µl from the last dilution.
6. Spread the diluted samples over the entire area of the test well starting with the highest dilution. Use a new stirrer for each sample.
7. Gently shake the antigen add 1 free-falling drop to each well using the antigen delivery needle provided. Carefully mix the antigen suspension and serum. Spread to cover the area of the well. Use a new stirrer for each sample.
8. Place the card on the mechanical rotator under a humidity cover and rotate for 8 minutes. If a mechanical rotator is not available rotate the card by hand with a steady circular motion for 2 minutes, then place it in a moist chamber containing wet tissue or filter-paper for 6 minutes. Remove the card and rotate briefly to obtain the final reading. Take care to avoid cross-contamination of samples.
9. Remove the card from the rotator and examine it macroscopically in a good light. The highest dilution to contain macroscopic agglutination is the titre of the sample.
10. If the sample is positive at 1:32, the dilution series should be extended. Prepare a 1:16 dilution in saline (0.85%) and perform a serial dilution series as described previously.

Fluorescent treponemal antibody absorption test (FTA-Abs)

The antigen used for the FTA-Abs test consists of *Treponema pallidum* (Nichols strain) which is fixed with acetone on a slide. Lyophilized *T. pallidum* cells reconstituted in saline can also be used. Inactivated patient serum is incubated with a sorbent consisting of Reiter treponemes for absorption of nonspecific treponemal group antibodies. After absorption, patient serum is added to the slide. Specific antibodies in the serum bind to the surface of the treponemal cells. After rinsing, a conjugate of antihuman antibodies with a fluorescent stain (fluorescein isothiocyanate) is added to the treponemes. The conjugate will bind to the antibodies that have bound to the treponemes and can be visualized by fluorescence microscopy.

Reactivity can be observed three weeks after infection and is permanent in untreated patients. It may be observed several years after successful drug treatment in the early phase of the disease and may be permanent in patients who receive adequate drug therapy only in the late phase of the disease. A positive reaction in the FTA-Abs test indicates a high probability of syphilitic infection. False-negative results are exceptional and may be due to poor quality antigen. False-positive results may be caused by group antibodies that were not eliminated during absorption procedures or by unsatisfactory reagents. False-positive results have also been reported in patients with

hepatic cirrhosis, balanitis, collagenosis, herpes gestationis, lupus erythematosus, and very occasionally in pregnant women and healthy persons, for unknown reasons.

Cross-reactivity between *T. pallidum* and *Borrelia burgdorferi* (Lyme disease) has been demonstrated. In particular, specimens with a disproportionately high antibody titre in an FTA-Abs test, compared with other serological syphilis tests, should be tested for antibodies against *Borrelia*.

The main advantages of the FTA-Abs test is its high specificity and sensitivity as well as the early onset of reactivity. The results are reliable and may be decisive in doubtful cases. However, the FTA-Abs test is time-consuming and expensive; it requires highly trained personnel to carry it out and to read the results. It should therefore be used only as a confirmatory test in cases where the diagnosis is in doubt.

Materials and reagents provided in the FTA-Abs test kit

Buffer solution
Control sera, positive and negative
Fluorescein-labelled antihuman immunoglobulin (conjugate)
Lyophilized extract of Reiter treponemes (sorbent)
T. pallidum smears fixed to slides

Additional materials and reagents required for the FTA-Abs test

Coverslips
Fluorescence microscope with UV illumination (×40 objective)
Mounting medium
Phosphate-buffered saline, pH 7.2 (PBS)
Tween 80 (2%)-PBS

FTA-Abs test

1. Remove the reagent kit from the refrigerator and allow the reagents to warm to room temperature.
2. Allow the required number of slides to warm to room temperature for 15 minutes.
3. Dilute 50 µl of serum with 0.2 ml of sorbent and mix. Dilute positive control and negative control sera in parallel, 1:5 in buffer solution and 1:5 in sorbent.
4. Cover the smear on one slide with 10 µl of buffer solution (conjugate control) and the smear on a second slide with 10 µl of sorbent (sorbent control).
5. On the remaining slides cover the smears with 10 µl each of serum dilution and control serum dilutions.
6. Place the slides in a moist chamber for 30 minutes at 37 °C. Wash the slides for 5 minutes in Tween-PBS solution. Repeat each wash. Rinse in distilled water, then drain and leave to dry in a slide box.
7. Cover the smears on all slides with 10 µl of antihuman immunoglobulin diluted in buffer solution according to the manufacturer's instructions.
8. Place the slides in a moist chamber for 30 minutes at 37 °C. Wash the slides for 5 minutes in Tween-PBS. Repeat each wash. Rinse in distilled water, then drain and leave to dry in a slide box.
9. Cover each smear with 2 drops of mounting medium and a coverslip.
10. Check that the conjugate, sorbent and negative serum controls do not fluoresce:

- No fluorescence or slightly greenish treponemes: negative reaction
- Varying degree of green fluorescence: positive reaction

The degree of fluorescence can be graded as:

Non-reactive	0
Borderline	1+, 2+, 3+
Reactive	4+

A reaction of 2+ or greater is regarded as indicative of *T. pallidum* infection.

Febrile agglutinins tests

Physicians who treat patients with inexplicable fever often request a series of serological tests, collectively known as febrile agglutinins tests, to demonstrate infections with *Brucella* (Wright test), *Salmonella typhi* (Widal test) and some rickettsias (Weil–Felix reaction). The tests measure agglutinating antibodies directed against an O surface antigen and/or an H flagella antigen of the suspected organism, or, in the case of the Weil–Felix reaction, a cross-reacting surface antigen of two strains of *Proteus vulgaris*.

The Widal and Weil–Felix tests are no longer recommended for the screening of patients as they pose a considerable number of technical and interpretational problems. A negative reaction will not exclude active infection as the infection may be in the incubation period and the patient has not yet produced detectable antibodies against the organism. The prozone phenomenon also results in a negative reaction; this can be prevented by using serial dilutions of the serum. A positive reaction with a given antigen may not be diagnostic, as the patient may exhibit a rise in heterologous agglutinins during the course of the illness. Such reactions are known as nonspecific anamnestic reactions because the patient has responded to an antigenic stimulus with production of nonspecific agglutinins. This makes serological diagnosis based on a single high antibody titre too uncertain, and only seroconversion with a fourfold or greater rise in titre on serial dilutions of sera should be accepted as an indication of a recent infection.

Most patients with acute brucellosis will have an agglutinin titre of 1:320 or greater by the end of the second week of illness. Even one year after treatment, 20% of patients will continue to have a significant *Brucella* agglutinin titre. High *Brucella* agglutinin titres have also been recorded in patients with *Francisella tularensis* and *Yersinia entero-colitica* infections, and in patients who have recently had a *Brucella* or cholera vaccination or been tested with brucellergin skin test. They have also occasionally been recorded in abattoir workers.

Because blood culture may not demonstrate the organism for many weeks, if ever, the *Brucella* agglutinin determinations can support a presumptive diagnosis of acute brucellosis. Isolation of the organism, usually from blood, provides definitive proof of infection. However, in suspected cases where blood culture does not show infection, sternal bone marrow aspirate may be cultured to confirm the diagnosis. Patients with localized brucellosis may be afebrile and may not have significant levels of *Brucella* agglutinin titre. In these cases, the infection should be suspected on epidemiological grounds and by detection of calcified lymph nodes on X-ray, but the diagnosis should be confirmed by culture.

The rapid slide test is a screening test designed to detect agglutinins, whereas the tube test is a confirmatory test designed to measure the agglutinin quantitatively. Any positive result obtained with the slide test should be verified with the tube test.

Materials and reagents provided in the febrile agglutinins test kit

Antigen suspensions (*B. abortus*, *B. melitensis*) in bottles fitted with droppers
Negative control serum
Positive control serum

Additional materials and reagents required for the febrile agglutinins test

Applicator sticks
Glass plate
Grease pencil
Pipettes, 50–1000 µl
Ruler
Sterile saline (0.85%)
Test-tubes and test-tube racks
Timer
Water-bath, temperature-controlled

Febrile agglutinins slide test

This test is preferred to the tube test as it is less complicated.

1. The serum sample must be clear and free of visible fat. It should not show haemolysis or be contaminated by bacteria. It should not be heat inactivated, as this may destroy some of the thermolabile agglutinins.
2. Prepare the glass plate by drawing up rows of 2.5-cm squares with the ruler and the grease pencil. Each row of 5 squares is sufficient to test one antigen against serum dilutions up to 1:320.
3. Use a 0.2 ml pipette to add 0.08, 0.04, 0.02, 0.01, and 0.005 ml of serum to a row of squares on the glass plate.
4. Place 1 drop of the appropriate well-mixed antigen suspension for the slide test on each drop of serum.
5. Mix the serum–antigen mixture with an applicator stick, starting from the highest serum dilution. The final dilutions are correlated approximately to the macroscopic tube test dilutions and are counted as 1:20, 1:40, 1:80, 1:160 and 1:320, respectively.
6. Hold the glass plate with both hands and gently rotate it 15–20 times. Examine the serum–antigen mixture macroscopically for agglutination within 1 minute in a good light. Reactions occurring later may be due to the reactants drying on the slide and should be verified with the tube test.
7. Record results as follows:

Complete agglutination	4+
Approximately 75% of the cells are clumped	3+
Approximately 50% of the cells are clumped	2+
Approximately 25% of the cells are clumped	1+
Trace or no agglutination	—

Febrile agglutinins tube test

Prepare serial dilutions of serum and the control sera in the following manner:

1. Place 8 test-tubes in a rack for each serum to be tested.
2. Pipette 1.9 ml of saline (0.85%) into the first tube of each row and 0.5 ml into each of the remaining tubes.
3. Add 0.1 ml of the serum to tube 1 containing 1.9 ml of saline.
4. Mix well with a pipette and transfer 0.5 ml to tube 2. Mix thoroughly.
5. Continue down the row of rubes adding 1 drop of serum dilution until tube 7. Mix thoroughly. Discard 0.5 ml from tube 7 after mixing thoroughly. Tube 8 is the antigen control tube.
6. Add 0.5 ml of the respective antigen to each of the 8 tubes. Shake the racks to mix the antigen and antiserum. The resultant dilutions are 1 : 20 through 1 : 1280, respectively.
7. Incubate in a water-bath at 37 °C for 48 hours or according to the manufacturer's instructions.
8. Examine the tubes macroscopically for agglutination within 1 minute in a good light against a black background. The tubes should not be shaken. Positive reactions show obvious agglutination (granulation); negative reactions show a cloudy suspension without agglutination. The highest degree of dilution of serum in a tube showing agglutination is the titre.

Discard an antigen if it does not agglutinate with a known positive control serum, or if it agglutinates with a known negative control serum.

Agglutinins may be found in healthy individuals, and single sera with titres of less than 80 are of doubtful significance. False-positive results may occur with sera from patients infected with *Francisella tularensis* or vaccinated against *Vibrio cholerae*. It is not possible to differentiate between *B. abortus* and *B. melitensis* infections using this test.

Antistreptolysin O (ASO) test

Streptococcal infections are very common in all populations, and a high percentage of people will have antibodies against streptococci. The β-haemolytic group A streptococci produce two haemolysins: oxygen-labile streptolysin O and oxygen-stable haemolysin S. Only reduced (non-oxidized) streptolysin O is immunogenic and is used for the test. The antistreptolysin O test is based on the fact that patients with *Streptococcus pyogenes* (group A streptococcal) infections develop antibodies that inhibit the haemolytic activity of streptolysin O. The antibodies are usually long-lasting and a single increased titre is not an indication of a current infection. Only a fourfold or greater rise in titre on successive serum samples taken 10–14 days apart should be considered indicative of recent infection. This test is mainly used in the diagnosis of acute rheumatic fever, acute glomerulonephritis and other post-streptococcal diseases.

There are two types of commercial antistreptolysin O test kits:

- The ASO latex slide agglutination test is used to screen sera to identify those with raised ASO titres (200 IU or higher).
- The ASO tube test is a haemolysis inhibition test that is used to determine ASO antibody titre in serum samples that are positive in the ASO latex slide agglutination test. A titre of less than 50 IU does not confirm the diagnosis of acute rheumatic fever.

Materials and reagents provided in the ASO latex slide agglutination test kit

Disposable cards, with 6 wells each
Disposable dropper
Positive control serum
Sensitized latex reagent (with streptolysin O)

Additional materials required for the ASO latex slide agglutination test

Applicator sticks

ASO latex slide agglutination test

1. Dilute the serum 1:20.
2. Place 1 drop of the serum solution in a well on the disposable card.
3. Use a new dropper to add 1 drop of sensitized latex reagent.
4. Use an applicator stick to mix the two drops and spread them over the entire well.
5. Examine for agglutination within 2 minutes.

A positive reaction appears as a fine flocculation (agglutination) within 2 minutes.
A negative reaction shows no agglutination.

If the flocculation appears within 2 minutes, the serum should be titrated with the antistreptolysin O tube test.

Materials and reagents provided in the ASO tube test kit

Positive control serum
Reduced streptolysin O antigen (dried preparation)
Sheep red blood cells
Standard antistreptolysin O antibody (dried preparation, 20 IU/bottle)
Streptolysin O buffer (25× concentrated solution)

Additional materials required for the ASO tube test

Distilled water
Pipettes (1 ml, 2 ml)
Test-tubes
Water-bath

ASO tube test

1. Reconstitute the reduced streptolysin O antigen (dried preparation) with the appropriate volume of distilled water (stated on the bottle label) to give a potency of 2 IU equivalent per ml. The solution should be used within 6 hours of reconstitution as it does not contain preservatives.
2. Reconstitute the standard antistreptolysin O antibody (dried preparation, 20 IU/bottle) with 10 ml of streptolysin O buffer. The solution can be stored for six months at 4 °C provided it does not become contaminated.
3. Dilute the streptolysin O buffer (25× concentrated solution) with 480 ml of distilled water before use. Diluted buffer should be discarded after one week.

4. Wash and centrifuge 1 ml of sheep red blood cells three times in streptolysin O buffer and pipette the supernatant fluid off. Add streptolysin O buffer to give an 8% cell suspension.
5. Allow the reagents and serum samples to reach room temperature.
6. Make a 1:10 dilution of patient serum in a test-tube (0.1 ml serum + 0.9 ml streptolysin O buffer). Prepare 2 master dilutions from the 1:10 dilution as shown in the table below:

Serum	Buffer	Dilution
0.2 ml (1:10)	1.8 ml	1:100
1.0 ml (1:100)	0.5 ml	1:150

7. Prepare the following dilution series with streptolysin O buffer for each of these master dilutions:

Tube	Serum	Buffer	Dilution	Reduced streptolysin O
1	0.2 ml (1:10)	0.8 ml	1:50	0.5 ml
2	0.5 ml (1:100)	0.5 ml	1:200	0.5 ml
3	0.5 ml (1:200)	0.5 ml	1:400	0.5 ml
4	0.5 ml (1:400)	0.5 ml	1:800	0.5 ml
5	0.5 ml (1:150)	0.5 ml	1:300	0.5 ml
6	0.5 ml (1:300)	0.5 ml	1:600	0.5 ml
7	0	1.5 ml	—	0
8	0	1.0 ml	—	0.5 ml

8. Rearrange the tubes in rising dilutions: 1:50, 1:200, 1:300, 1:400, 1:600, 1:800.
9. Add 1.5 ml of buffer to control tube 7 and 1 ml of buffer to control tube 8.
10. Add 0.5 ml of reduced streptolysin O to all test-tubes, except control tube 7.
11. Mix and refrigerate at 4 °C for two hours to allow the antibody–antigen reaction to take place.
12. Add 0.5 ml of the 8% cell suspension to each tube, including control tubes 7 and 8, mix and incubate in a water-bath at 37 °C for 30 minutes.
13. Centrifuge the tubes at 1000 *g* for 2 minutes and observe for haemolysis. Control tube 7 should show no haemolysis and control tube 8 should be completely haemolysed.
14. The ASO titre is determined as the highest dilution showing no sign of haemolysis:
    - If there is haemolysis in all tubes, report the result as "ASO titre less than 200 IU".
    - If there is no haemolysis in the tubes with a higher serum dilution, report the result as "ASO reactive with the titre".

Bacterial antigen tests

Latex agglutination and coagglutination tests for bacterial antigens are used to identify microorganisms or their antigens in cultures or in clinical specimens. Latex agglutination tests use polymer particles as solid-phase support; coagglutination tests use red blood cells as solid-phase support. Latex agglutination tests are available to detect a number of different polysaccharide anti-

gens of bacteria that cause meningitis including *Haemophilus influenzae* (type b), *S. pneumoniae* (omnivalent), *N. meningitidis* (group A, B, C, Y and W135), *E. coli* (type K1), and *S. agalactiae* (group B). Latex agglutination tests are useful for identifying streptococci from Lancefield groups A, B, C, D, F and G. Moreover, latex agglutinating sera are available for use in qualitative slide agglutination tests and quantitative tube agglutination tests for serological identification and typing of *Streptococcus* groups A–D, *N. meningitidis, H. influenzae, Salmonella, Shigella, Vibrio cholerae,* etc.

A threshold level of the antigen is required to detect polysaccharide antigens in clinical samples. The threshold level is usually exceeded if organisms can be seen in Gram-stained preparations, although this may not always be the case. The number of bacteria found in the cerebrospinal fluid of patients with *N. meningitidis* infection is significantly lower that that for other types of *Neisseria* spp. infection and therefore the threshold level of polysaccharide antigens is reached less frequently.

Several serogroups of *N. meningitidis* may cause meningitis. While serogroups A, C, Y, and W135 each have a stable antigen that can be detected with a single polyvalent reagent, the serogroup B antigen is relatively unstable and considerably more difficult to detect. It is also indistinguishable from the K1 polysaccharide antigen of *E. coli*, which is the main cause of *E. coli* meningitis in newborn babies. However, if the Gram-stained preparation demonstrates Gram-negative diplococci, infection is most likely to be due to *N. meningitidis* group B; if the Gram-stained preparation demonstrates Gram-negative rods, infection is probably due to *E. coli*.

For some of the tests a polysaccharide antigen is extracted from the organism before testing. This extraction may be carried out chemically or enzymatically. Four different tests can be used for the detection of the antigen:

- In the slide-agglutination tests, the antigen is added to latex particles or staphylococcal cells coated with specific antibodies. The mixture is rotated by hand with a steady circular motion for 1–2 minutes and the reaction is observed macroscopically for agglutination.
- In the ELISA tests, a solution of sample and antigen is first passed over a membrane coated with an antibody (this antibody is usually monoclonal). Subsequently, the membrane is covered with a solution of a second (monoclonal) antibody conjugated with an enzyme. The presence of the antigen–antibody enzyme complex on the membrane is detected by its reaction with a chromogenic substrate that is added in solution.
- In the gold immunoassay, a solution of sample and antigen is left to diffuse on a membrane, which is then examined.
- In the optical immunoassay, antibodies are attached to a silicon wafer with reflective properties. When the antigen reacts with the specific antibody on the wafer, a change in the surface layer occurs, which causes a change in the light reflection.

Typical procedures for latex agglutination or coagglutination tests

1. *For cell-bound antigens*: use a sterilized loop to transfer well-isolated colonies to a drop of saline on a slide and mix carefully to obtain a slightly opalescent emulsion. If the saline emulsion shows clumping, it usually indicates a rough (R) strain instead of a smooth (S) strain, and the cells will fail to agglutinate with the antibody. If no clumping appears, add the reagent and proceed from step 4.

For extracted antigen: use a sterilized loop to transfer well-isolated colonies to a small test-tube containing extraction solution and emulsify them. Incubate the emulsion at 35 °C or according to the manufacturer's instructions. *For clinical specimens (CSF, urine)*: heat the specimen to boiling-point or according to the manufacturer's instructions. Cool and centrifuge at 2000 *g* for 5–10 minutes.

2. Place 1 drop of the antibody-coated particles on the slide.
3. Place 1 drop of the antigen suspension beside the antibody-coated particles.
4. Mix the 2 drops on the slide and spread in a square.
5. Rotate the slide by hand in a steady circular motion for 1 minute. Take care that the mixture does not spill outside the boundaries of the square.
6. Examine the slide macroscopically for agglutination after the time specified in the manufacturer's instructions. For best visibility hold the slide near a bright light and view against a dark background.
7. A positive result is recorded when the antigen mixed with the antibody shows agglutination, i.e. the suspension shows clumping or is granular to the point of curdling. Agglutination is best seen by tilting the slide slightly so that the fluid drains down towards the lower boundary of the square.

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Part II

Essential media and reagents

Introduction

With just a few diagnostic materials, a laboratory can make an important contribution to individual patient care through accurate etiological diagnosis. In most developing countries bacteriological laboratory practice is hampered by a shortage of culture media and basic reagents, which are very costly to import. However, the number of culture media and reagents that have to be purchased can be reduced to the essential ones, through rational selection, as has been the case with essential drug lists. Additionally, some simple media and reagents can be produced or prepared locally. Application of these two approaches would greatly reduce the necessity for foreign exchange and make more readily available the laboratory material necessary for patient care and epidemiological studies.

This chapter has been prepared to enable health laboratory managers to concentrate their resources on the most relevant media and reagents. It comprises two sections both composed of a series of lists.

Pathogens, media and diagnostic reagents

Expected pathogens

Pathogens are listed according to a number of factors:

— frequency of isolation,
— clinical relevance,
— severity of disease,
— epidemic potential,
— cost–benefit ratio of isolation and/or identification.

The listing is by no means absolute and will vary from country to country or from laboratory to laboratory, depending on the local disease pattern, the laboratory capacity, and the resources available.

Media and diagnostic reagent priority gradings

A certain degree of flexibility has been incorporated by adopting a priority grading for media and diagnostic reagents as follows:

Grade 1: High priority
Grade 2: Intermediate priority
Grade 3: Low priority

Media and diagnostic reagents are priority graded according to the listing of the pathogens for which they are used for isolation and identification. However, there may be differences. If the medium is used broadly for more than one pathogen, it may score higher than any one pathogen for which it is used.

Grade 1: High priority media and diagnostic reagents should be available in all laboratories that practise general diagnostic bacteriology. They are most often for general purpose use, easy to prepare and few in number.

Grade 2: Intermediate priority media and diagnostic reagents are additional useful substances that make laboratory diagnosis more complete and more useful for epidemiological studies, although they may not be essential for direct patient care, e.g. the grouping antisera for meningococci.

Grade 3: Low priority media and diagnostic reagents are substances that are of value only occasionally for patient care, but which are useful for teaching, research, and special investigations by a reference laboratory. This category applies to media and diagnostic reagents that are too expensive for general use, or that are needed for the isolation and identification of organisms that occur rarely or are difficult to isolate and therefore often not cost-effective.

For common laboratory investigations, the listed pathogens, and the required media and diagnostic reagents, together with a suggested priority grading, are to be found below. The priority grading should be adapted for each laboratory according to local circumstances.

Blood

Expected pathogens

Bacteroides fragilis
Brucella
Burkholderia pseudomallei
Candida albicans and *Cryptococcus neoformans*
Haemophilus influenzae
Neisseria meningitidis
Non-fermenters other than *Pseudomonas aeruginosa*
Other Enterobacteriaceae
Pseudomonas aeruginosa
Salmonella typhi and non-*typhi*
Staphylococcus aureus
Streptococci (*S. pyogenes*, *S. pneumoniae*, viridans streptococci)

Media and diagnostic reagents

Blood-culture broth

	Priority grading
Tryptic soy broth (TSB) can be replaced by any rich broth, e.g. brain–heart infusion broth, addition of sodium polyanethol sulfonate (SPS), 0.25 g/l, optional	1
"Anaerobic" blood culture broth: thioglycollate broth *or* Schaedler broth *or* Wilkins–Chalgren anaerobe broth	2

Isolation media

Subculture on blood agar, chocolate agar and MacConkey agar	1

Diagnostic reagents

Bacitracin disc	1
Coagulase plasma	1
β-Lactamase test reagent	1
Optochin disc	1
Oxidase reagent	1
Salmonella agglutinating antisera	1
V and XV factors	2
Haemophilus influenzae type b antiserum	3
Neisseria meningitidis agglutinating serum (polyvalent and specific groups A, B, C)	3

Cerebrospinal fluid

Expected pathogens

Cryptococcus neoformans
Enterobacteriaceae
Haemophilus influenzae
Listeria monocytogenes

Mycobacterium tuberculosis
Neisseria meningitidis
Streptococcus agalactiae
Streptococcus pneumoniae

Media and diagnostic reagents

Isolation media

	Priority grading
Blood agar (with streak of *Staphylococcus*)	1
Chocolate agar	1
MacConkey agar	1
Löwenstein–Jensen medium	2
Sabouraud dextrose agar	2

Diagnostic reagents

India ink	1
β-Lactamase test reagent	1
Optochin disc	1
Oxidase reagent	1
V and XV factors	2
Haemophilus influenzae type b antiserum	3
Neisseria meningitidis agglutinating serum (polyvalent and specific groups A, B, C)	3

Rapid diagnostic tests

Test kit for rapid diagnosis of bacteria causing meningitis	3

Urine

Expected pathogens

Candida albicans
Enterococci
Escherichia coli
Mycobacterium tuberculosis
Other Enterobacteriaceae
Other staphylococci
Pseudomonas and other non-fermenters
Staphylococcus saprophyticus

Media and diagnostic reagents

Isolation and quantitative media

	Priority grading
Blood agar	1
Brolacin agar (can be replaced by purple lactose agar,	

MacConkey agar, agar without crystal violet, or eosin methylene-blue agar)	1
CLED agar	1

Identification media and diagnostic reagents

β-Glucuronidase tablet (PGUA) for identification of *E. coli*	1
For Gram-negative rods:	
Kligler iron agar (KIA)	1
Kovacs reagent for indole	1
motility–indole–urease (MIU) medium	1
oxidase reagent	1
lysine–decarboxylase broth (Möller)	2
ONPG test	2
Simmons citrate agar	2
For staphylococci and enterococci	
catalase test (H_2O_2)	1
coagulase plasma	1
bile–aesculin agar (for enterococci)	2
novobiocin (5 µg) disc for differentiating negative-coagulase staphylococci	3

Stool

Expected pathogens

Aeromonas and *Plesiomonas*
Campylobacter spp.
Escherichia coli (enteropathogenic, enterotoxigenic, enteroinvasive, and enterohaemorrhagic)
Non-typhoid *Salmonellae* spp. and *Edwardsiella*
Salmonella typhi and *S. paratyphi*
Shigella
Vibrio cholerae serogroup O1, non-cholerae vibrios
Yersinia enterocolitica

Media and diagnostic reagents

Transport media

	Priority grading
Cary–Blair medium (for all pathogens)	1
Buffered glycerol saline (not for *Vibrio* or *Campylobacter*)	2

Enrichment media

Selenite F broth	1
Alkaline peptone water	2

Isolation media

Deoxycholate–citrate agar (can be replaced by *Salmonella-Shigella* agar, xylose–lysine–deoxycholate agar (XLD)	1
MacConkey agar (with crystal violet)	1
TCBS agar	1
Campylobacter medium: Columbia agar base or any blood agar base with lysed blood and antibiotic supplement, or charcoal-based media	2

Preliminary media and diagnostic reagents

Kligler iron agar (KIA) (may be replaced by triple sugar iron agar (TSI) but only for enteric pathogens)	1
Kovacs reagent for indole	1
Motility–indole–urease (MIU) medium (may be replaced by motility test medium + peptone urea broth)	1
Oxidase reagent	1

Specific media and diagnostic reagents

Andrade peptone water (or phenol red broth base)	2
Lysine–decarboxylase broth (Möller)	2
ONPG test	2
Simmons citrate agar	2
Vibriostatic compound O:129 disc	2

Agglutinating antisera

		Priority grading
Salmonella:	O antiserum polyvalent (A–I and Vi)	1
	O-factor antisera: O:2 (A), O:4 (B), O:9 (D), Vi	2
	H-factor antisera: H:a, H:b, H:d, H:i, H:m, H:2	3
	phase inversion H-antisera: H:b, H:i, H:1,2	3
Shigella:	*dysenteriae* polyvalent, *flexneri* polyvalent, *boydii* polyvalent, *sonnei* polyvalent	1
	dysenteriae type 1 (Shiga)	1
Vibrio cholerae:	O1 antiserum polyvalent	1
	subtypes B (Ogawa), C (Inaba), O:139	3
Haemophilus influenzae	type b	3
Neisseria meningitidis	polyvalent	3
	single factor A, B, C	3

Upper respiratory tract

Expected pathogens

Candida albicans (oropharynx)
Corynebacterium diphtheriae (throat and nose)
Haemophilus influenzae (ear and sinus)
Moraxella catarrhalis (ear and sinus)
Neisseria meningitidis
Pseudomonas

Staphyloccoccus aureus (ear and sinus)
Streptococcus pneumoniae (ear and sinus)
Streptococcus pyogenes (group A, throat)

Media and diagnostic reagents

Isolation media

	Priority grading
Blood agar (prepared from a glucose-free base)	1
Chocolate agar	2
Löffler coagulated serum or Dorset egg medium	2
Tellurite blood agar	2
Modified Thayer–Martin medium (for gonococci and meningococci)	3

Diagnostic reagents

Bacitracin disc	1
Catalase and coagulase reagents	1
Optochin disc	1
Carbohydrate degradation media for *Neisseria* spp.	2
Oxidase reagent	2
V and XV factors (discs or strips)	2
Tributyrin	3

Rapid diagnostic tests

Grouping kit for haemolytic streptococci	3

Lower respiratory tract

Expected pathogens

Candida albicans
Enterobacteriaceae
Haemophilus influenzae
Klebsiella pneumoniae
Moraxella catarrhalis
Mycobacterium tuberculosis
Staphylococcus aureus
Streptococcus pneumoniae

Media and diagnostic reagents

Isolation media

	Priority grading
Blood agar	1
Chocolate agar	1
MacConkey agar	1
Löwenstein–Jensen medium	2

Sabouraud dextrose agar	3
Selective blood agar for *Haemophilus* (bacitracin or vancomycin)	3

Diagnostic reagents

Coagulase plasma	1
Optochin disc	1
Oxidase reagent	2
V and XV factors (discs or strips)	2
Tributyrin	3

Urogenital specimens for exclusion of sexually transmitted diseases (STDs)

Expected pathogens

Candida albicans (microscopic examination)
Chlamydia trachomatis
Gardnerella vaginalis (microscopic examination)[1]
Haemophilus ducreyi
Neisseria gonorrhoeae
Treponema pallidum (dark-field microscopy)

Media and diagnostic reagents

Transport media

	Priority grading
Amies transport medium or Stuart transport medium	1

Isolation media

Modified Thayer–Martin (MTM) medium or New York City (NYC) medium	1
Mueller–Hinton chocolated horse-blood agar + vancomycin + IsoVitaleX for *H. ducreyi*	3

Identification reagents

Nitrocefin test or other β-lactamase test reagent	1
Oxidase reagent	1

Pus and exudates

Expected pathogens

Bacillus anthracis
Bacteroides and other strict anaerobes

[1] *Gardnerella vaginalis* is an indicator organism for vaginosis, but not a pathogen.

Clostridium perfringens
Enterobacteriaceae
Mycobacterium tuberculosis, M. ulcerans
Other *Mycobacterium* spp.
Pasteurella multocida
Pseudomonas and other non-fermenters
Staphylococcus aureus
Streptococcus pyogenes
Streptococcus (other species)

Media and diagnostic reagents

Isolation media

	Priority grading
Blood agar	1
MacConkey agar	1
Mannitol salt agar	2
Thioglycollate broth (with indicator) (can be replaced by cooked meat medium, Schaedler broth, Wilkins–Chalgren broth)	2
Tryptic soy broth (TSB)	2

Diagnostic reagents

Catalase test (H_2O_2)	1
Coagulase plasma	1
Oxidase reagent	1
Hydrogen generator for anaerobic jar	2

List of recommended media and diagnostic reagents for the intermediate microbiological laboratory

Culture media

Recommended medium	Alternatives	Priority grading
Bile–aesculin agar		1
Blood agar (see tryptic soy agar)		1
Brolacin agar	purple lactose agar, CLED agar	1
Kligler iron agar (KIA)		1
Löffler coagulated serum	Dorset egg medium	1
Löwenstein–Jensen medium		1
MacConkey agar (with crystal violet)	eosin methylene-blue agar	1
MacConkey agar (without crystal violet)		1
Motility–indole–urease (MIU) medium	motility test medium + urea broth + peptone (tryptone) water	1
Mueller–Hinton agar		1
Sabouraud dextrose agar		1
Deoxycholate citrate agar	*Salmonella–Shigella* (SS) agar	1
Tryptic soy agar (TSA)	Columbia agar	1

Recommended medium	Alternatives	Priority grading
Tryptic soy broth (TSB)	brain–heart infusion broth	1
TCBS		1
Transport medium (Amies)	transport medium (Stuart or Cary–Blair)	1
Andrade peptone water	phenol red broth	2
Decarboxylase broth (Möller)		2
Mannitol salt agar (MSA)		2
Selenite F broth		2
Simmons citrate agar		2
Thioglycollate medium (with indicator)	Schaedler broth, Wilkens–Chalgren anaerobe broth, cooked meat medium	2
DNase agar		3

Inhibitors or antimicrobials for use in media or as reagents

Chloramphenicol (for isolation of fungi)	1
Gonococcal antimicrobial supplement: vancomycin, colistin, nystatin (trimethoprim): VCN (VCNT)	1
Campylobacter antimicrobial supplement	2
Tellurite solution (for isolation of *Corynebacterium diphtheriae*)	2
Bacitracin (for isolation of *Haemophilus* spp.)	3
Vancomycin (for isolation of *Haemophilus ducreyi* or *H. influenzae*)	3

Enrichments for culture media

IsoVitaleX (Polyvitex, Vitox, supplement B, supplement VX, supplement CVA)	2
Sodium polyanethol sulfonate (SPS)	3

Diagnostic discs, tablets or strips

Bacitracin disc	1
Nitrocefin disc (Cefinase) or reagent	1
ONPG test	1
Optochin disc	1
Oxidase reagent	1
PGUA (β-glucuronidase)	1
V and XV factors	2
Novobiocin (5μg) disc	3
PYR test	3
Tributyrin	3
Vibriostatic compound O:129 disc	3

Diagnostic kits

Rapid serodiagnostic kit for identification of bacteria causing meningitis	3
Serological grouping kit for haemolytic streptococci	3

Miscellaneous diagnostic reagents

Barium sulfate standard (for Kirby–Bauer method)	1
Gram-stain reagents	1
Hydrogen peroxide (H_2O_2) (catalase)	1
Kovacs reagent (for indole)	1
Oxidase reagent (dimethyl-*p*-phenylenediamine)	1
Plasma (for coagulase test and germ-tube test)	1
Ziehl–Neelsen stain	1
Buffered glycerol–saline (for transportation of stool)	2
Carbohydrates: glucose, lactose, maltose, mannitol, sucrose	2
Hydrogen generator for anaerobic jar	2
India ink (for capsule detection)	2
Lysine (for decarboxylase test)	2

Sensitivity-testing discs

Antimicrobials included in the WHO list of essential drugs (2002)

amoxicillin
ampicillin
benzylpenicillin
chloramphenicol
ciprofloxazin
co-trimoxazole (sulfamethoxazole–trimethoprim)
cloxacillin
erythromycin
gentamicin
kanamycin
nalidixic acid
nitrofurantoin
sulfonamide
tetracycline (or doxycycline)
trimethoprim

Reserved antimicrobials

amoxy-clav
amikacin
cefalotin
cefazolin
cefotaxime
ceftazidime
ceftriaxone
cefuroxime
ciprofloxacin or other fluoquinolones
clindamycin
piperacillin
vancomycin

Agglutinating antisera

		Priority grading
Salmonella:	O antiserum polyvalent (A–I and Vi)	1
	O-factor antisera: O:2 (A), O:4 (B), O:9 (D), Vi	2
	H-factor antisera: H:a, H:b, H:d, H:i, H:m, H:2	3
	phase inversion H-antisera: H:b, H:i, H:1,2	3
Shigella:	*dysenteriae* polyvalent, *flexneri* polyvalent, *boydii* polyvalent, *sonnei* polyvalent	1
	dysenteriae type 1 (Shiga)	1
Vibrio cholerae:	O1 antiserum polyvalent	1
	subtypes B (Ogawa), C (Inaba), O:139	3
Haemophilus influenzae:	type b	3
Neisseria meningitidis:	polyvalent	3
	single factor A, B, C	3

Selected further reading

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Index

Note: Page numbers in bold type indicate main discussions.

Abscesses 86, 87, **89**, 93, 98
  liver 92
  lung **66–67**
Acid-fast stain, *see* Ziehl–Neelsen staining
Acinetobacter
  lwoffi 10–12, 24
  spp. 60, 70
Acquired immunodeficiency syndrome (AIDS) 20, **37**, 61, 76
Actinomyces israelii 90, 93
Actinomycosis 89, 90
Acute bronchitis 66
Aeromonas spp. 38, 53, 146
Aesculin agar, *see* Bile–aesculin agar
African trypanosomiasis 26
Agglutinating antisera **147**, **153**
Agglutination tests 28, 56–59, 63, **125–126**, **135–139**
Agglutinins tests, febrile **133–135**
AIDS, *see* Acquired immunodeficiency syndrome
Albert stain 64
Albumin, bovine 73
Alcohol 21
Alkaline bile salt agar 42
Alkaline peptone water 40–42, 146
American Type Culture Collection 14
Amies transport medium 40, 62, 77, 81, 149, 151
Amikacin 107, 110, 116, 152
Aminoglycosides 100, 109
Amoeba 26, 87, 92
Amoebic dysentery 41
Amoxicillin 109, 152
Amoxy-clav 107, 110, 116, 152
Amphotericin B 42, 78
Ampicillin 107, 109, 110, 116, 152
Anaemia, aplastic 108
Anaerobes 10, 80, 87, **98–102**
  necrotizing ulcerative pharyngitis 61
  stab cultures 15, 24
  surgical specimens 91, 93
  wound infections 88
Anaerobic jars **8**, 44
Andrade peptone water 64, 147, 151
Aneurysms, infected 20
Animal bites/scratches **88**, **96**
Anorectal specimens, collection 77
Anthrax (*see also Bacillus anthracis*) **96–97**
Antibiograms (*see also* Susceptibility tests)
  blood cultures **24**
Antibiotic discs, *see* Antimicrobial discs
Antibiotic susceptibility testing, *see* Susceptibility testing
Antibodies 122
Anticoagulant, blood collection **22**
Antigens 77, 122
  diagnostic **12–13**
  latex agglutination test 137, **138–139**
  VDRL test 128
Antimicrobial discs 13–14, 103, 104–105, **108–121**
  potency 119
  recommended **151**, **152**
Antimicrobial resistance 17, 62, 97, **101–121**, 108
Antimicrobial susceptibility, definition **104–105**
Antimicrobial susceptibility testing, *see* Susceptibility testing
Antimicrobials (*see also* Drugs)
  enteric disease therapy **39**
  generic names 108
  media/reagent use **151**
  susceptibility testing discs 103, 104–105, **108–121**, **151–152**
Antisera
  agglutinating **147**, **153**
  diagnostic **12–13**
  external quality assessment 17–18
Antistreptolysin O (ASO) test **135–137**
Arabinose 53
Arcobacter butzleri 39, 54
Argenine 38, 54
Arthritis 76, 93, 96
ASO, *see* Antistreptolysin O test
Aspirates 80, 86–87, 92
Autoclaves, quality control **8**

Bacillus anthracis 88, 91, 93, **96–97**, 149
Bacitracin 148, 151
Bacteraemia **20–21**, 24, 37, 101, 102
Bacteria, oxygen requirement **98**
Bacteriology, quality assurance **2–18**
Bacteriuria, screening test 32
Bacteroides
  fragilis 12, 21, 87, 88, 91, 144
    identification 99, **101**, 102
    stock strain 16
  melaninogenicus, *see Prevotella melaninogenica*
  spp. 98–99, 149
Balanitis 132
Balanoposthitis 76
Barium sulfate 12, 152
Bartholinitis 76
Bed sores 87, **89–90**
Benzalkonium chloride, *see* Zephiran
Benzylpenicillin 79, 97, 106–108, 110, 116, 152

Bile–aesculin agar 11, 23, 29, 64, 146, 150
Bile stimulation test 101
Bismuth sulfite agar (BSA) 44, 48
Bites, animal/human **88**, **96**
Blastomyces dermatitidis 92
Blind subcultures, blood samples **23**
Blood agar 27–29,
  anaerobic specimens 100
  Corynebacterium diphtheriae 64
  CSF specimens 27
  Haemophilus influenzae 29, 149
  priority grading 144–145, 148–150, 155
  sputum culture 69
  Streptococcus pyogenes culture **63**
  surgical specimens 92–93
Blood specimens **20–24**
  collection **20–22**
  culture 21, **22–24**
  diagnostic reagents/media **144**
  expected pathogens **144**
Body cavities, purulent exudates 87, **90**
Bordetella pertussis 126
Borrelia burgdorferi 132
Bottles, blood-culture **22–23**
Botulism, wound 88, 98
Brain heart infusion broth 144, 151
Branhamella catarrhalis, *see Moraxella catarrhalis*
Brolacin agar 145, 150
Bromothymol blue–lactose–cystine agar 34, 145, 150
Bronchitis **66**
Bronchopneumonia **67**
Broth
  anaerobes culture 99, 100, 101, 144, 151
  blood culture **22**
  thioglycollate 12, 23, 38, 92, 144, 150
  tryptic soy (TSB) 22, 27, 96, 144, 150, 151
  Wilkins–Chalgren 100, 144, 151
Brucella
  abortus 23, 135
  melitensis 133, 135
  spp. 16, 17, 23, 59, **133**, 135, 144
Brucellosis 20, **133**
BSA, *see* Bismuth sulfite agar
Buboes, inguinal 83
Burkholderia pseudomallei 17, 21, 144
Burns **87**, **89–90**

Calcium alginate 77
Calibrated loop technique, urine specimens **32–33**
Calymmatobacterium granulomatis 76, 82
CAMP test 23
  reverse 28, 64, **101–102**
Campylobacter
  antimicrobial supplement 151
  coli 38, 50, 54, 55
  fetus 54, 55
  hyointestinalis 54, 55
  jejuni 38, 50, 54, 55
  lari 54, 55
  media 147
  spp. 7, 41, 42, **50**, 98
    enteric infections 37, **38**, 146, 147
    identification **54**, **55**
    incubation **43–44**
  upsaliensis 54, 55
Cancer, cervical 76
Candida
  albicans 12, 21, 61, 144–149
    in sputum 69
    STDs 76, 79, 81, 82
    stock strain 10, 70, 71
  spp. 61, 62, 93
Candidiasis, oral 61–62
Candle jars 43, 69, 100
Carbapenem 97
Carbohydrate degradation 79, 148
Carbohydrates 152
Carbohydrate utilization test 28
Carbol fuchsin 50
Carbon dioxide 28, 77
Cardiolipin lecithin 126, 127
Carriers, upper respiratory tract infections 62
Cary–Blair transport medium 40–41, 146, 151
Castaneda bottle 23
Catalase test 13, 29, 46, 52, 64, 96, 146, 148, 150, 152
Causal agents
  bacteraemia **21**
  diarrhoeal disease **37–39**
  fungaemia **21**
  meningitis **25**
  pharyngitis **61–62**
  purulent exudates **87**
  STDs **76–77**
  tonsillitis **61**
CCFA, *see* Cefoxitin–cycloserine–fructose agar
Cefalexin 109
Cefalosporins 97, 109
Cefalotin 107, 109, 110, 116, 152
Cefalozin 110, 116, 152
Cefamandole 109
Cefamycin 109
Cefapirin 109
Cefotaxime 109, 116, 152
Cefoxitin 109
Cefoxitin–cycloserine–fructose (CCFA) agar 42, 50
Cefradine 109
Ceftazidime 107, 110, 116, 152
Ceftriaxone 107, 110, 116, 152
Cefuroxime 107, 109, 110, 152
Cellobiose 53
Cellulitis 93, 96
Centrifuges, quality control **8**
Cerebrospinal fluid (CSF) **25–29**
  diagnostic reagents/media **145**

diagnostic tests 29, 125, 127–129, 145
expected pathogens **144–145**
macroscopic inspection **26**
meningitis **27**
microscopic examination **26–28**
Neisseria meningitidis 138
pathogen identification 28–29
specimen collection/transport **25**
specimen culture **27–29**
susceptibility testing 29
Cervical cancer 76
Cervicitis 76, 79, 81
Cervicofacial actinomycosis 90
Cervicovaginal specimens (*see also* Sexually transmitted diseases)
collection **80**
culture **82**
interpretation **81–82**
microscopic examination **81**
transport 81
CFUs, *see* Colony-forming units
Chancre 84
Chancroid 76, 83, 85
Children
bacterial pharyngitis 61
diarrhoeal diseases 37
neonates 21, 25, 80, 82
surgical specimens 93
urine specimens **31**
Chlamydia
spp. 66, 83
trachomatis 76, 77, 79–80, 82, 83, 126, 149
Chloramphenicol 107, 108, 110, 116, 151, 152
Chlorhexidine 21
Chlortetracycline 108
Chocolate agar 11, 27–28, 69, 78, 145, 148
Cholera **38**, 41
Chorio-amnionitis 76, 80
Chromosal resistance 79
Chronic bronchitis 66
Ciprofloxacin 107, 110, 116, 152
Citrate 22, 25, 51
Citrobacter
freundii 10, 12, 17, 51
spp. 25
Classification, *Salmonella* spp. **54–55**
CLED agar, *see* Cystine–lactose–electrolyte-deficient agar
Clindamycin 107, 110, 116, 152
Clinical relevance **4–5**
Clostridium
bifermentans 42
botulinum 98
difficile **39**, 41, 42, 43, **50**
perfringens 10, 21, 88, **101–102**, 150
sordelli 42
spp. 21, 24, 42, 80, 98–99, 100
tetani 98
Cloxacillin 97, 108, 152
Clue cells 81
Coagulase plasma 13, 70, 144, 146, 149, 150
Coagulase test, *Staphylococcus aureus* **94–96**
Colistin 78, 151
Colitis 20, 38–39
Collagenosis 127, 132
Collection
anaerobic specimens **99**
anorectal specimens 77
blood specimens **20–22**
cervicovaginal specimens **80**
CSF specimens **25**
genital ulcer specimens **83**
pharyngeal specimens **62**
sputum specimens **68**
stool specimens **40**
surgical specimens **89–90**
urethral specimens **77–78**
urine specimens **30–32**
Colony-forming units (CFUs) 34–35
Concentration, minimal inhibitory (MIC) 5, 71, 104–105
Conjunctivitis 76, 80, 81
Containers, urine specimens 30
Contamination
anaerobic cultures 100
blood cultures **21**, **24**
Control strains
bacteria 14
susceptibility testing **110**, 120
Cooked meat broth, anaerobes 99, 101
Cooked meat medium 92, 150, 151
Corynebacterium
diphtheriae **61**, 65, 126, 147
carriers 62
culture/identification **64–65**
mitis 64
Coryneform rods 92
Cost–benefit ratio, diagnostic tests 2
Co-trimoxazole 107, 109, 110, 116, 152
Crepitation 90
Criteria, quality assurance **4–6**
Cross-reactivity 59, 132
Cross-resistance, staphylococci 97
Cryptococcus neoformans 4, 21, 25, 26, 28, 144
CSF, *see* Cerebrospinal fluid
Culture media (*see also* Media) **7–8**, **10–12**
blood specimens **22–23**
CSF specimens **27–28**
dehydrated **7–8**
ordering/storage **7–8**, **10**
performance tests **11–12**
preparation 8
priority grading 143, **150–151**
quality control **10–12**
selection **7**
stool specimens **41–42**, **44**, **48**
storage **7–8**, **10**
urine specimens 33–34

Cultures 13–15, 27, 29
anaerobic 61, 91, 93, **99–101**
blood specimens **22–24**
Candida albicans 82
cervicovaginal specimens 182
Corynebacterium diphtheriae **64–65**
CSF specimens **27–28**
external quality assessment 16, **17**
Gardnerella vaginalis 82
genital ulcer specimens **85**
Mycobacterium tuberculosis **72–75**
Neisseria gonorrhoeae **78–79**, **82**
preservation **14–16**
sputum specimens **69–70**
stab cultures 15, 24, 29
stock 10, **14–16**
Streptococcus pyogenes 63, **63**
surgical specimens **92–93**
susceptibility testing **120**
urine specimens **32–35**
URT specimens **63–65**
Cystine–lactose–electrolyte-deficient (CLED) agar 33, 146
Cystine trypticase agar (CTA) 15
Cystitis 30
Cytomegalovirus 76
Cytotoxin 39

DCA, *see* Deoxycholate citrate agar
Decarboxylase 11
Decarboxylase broth 151
Decontamination procedures, *Mycobacterium tuberculosis* cultures 67, **72–74**
Decubitus ulcers 87, **89–90**
Definitions
antimicrobial resistance/susceptibility **104–105**
quality assessment **4**
quality assurance terms **2**
quality control **3–4**
Dehydrated media **7–8**
Dehydrolase 11
Dental surgery 89
Deoxycholate citrate agar (DCA) 7, 11, 42, 44, 48, 147, 150
Dermatitis 76
Dextrose oxidation/fermentation (*see also* Glucose) 11
Diagnostic discs, *see* Antimicrobial discs
Diagnostic kits, recommended **151**
Diagnostic reagents, *see* Reagents
Diagnostic sensitivity **5–6**, 13, 83
Diagnostic specificity **6**, 13, 83
Diagnostic tests (*see also* Tests)
error sources 2
quality assurance **2–18**
Diarrhoeal diseases **37–39**, 41
Dicloxacillin 108
Diffusion test, antimicrobial resistance **104**
Dilution, serological tests 131–133, 137
Dilution test, antimicrobial resistance **103–104**
Dimethyl-*p*-phenylenediamine (oxidase reagent) 52
Diphtheria (*see also Corynebacterium diphtheriae*) 61, **64–65**
Disc-diffusion technique, *see* Kirby–Bauer disc-diffusion technique
Discs, antimicrobial 13–14, 103, 104–105, **107–121**, **151–152**
Disinfection, skin **21**, 25
Dispatch (*see also* Transport)
Mycobacterium tuberculosis cultures **75**
pharyngeal specimens **62**
sputum specimens **68**
Distilled water, serological tests 124
DNAse agar 94, 151
Donovanosis, *see* Granuloma inguinale
Dorset egg medium 64, 151
Doxycycline 152
Drugs (*see also* Antimicrobials)
nonproprietary names 108
susceptibility testing **107–109**
WHO list of essential 152
Dysenteriae polyvalent antiserum 147, 153
Dysentery 37–38, 41

Edwardsiella tarda 51, 146
Egg yolk agar base 64
Elek test (in-vitro toxigenic test) 38, 64
ELISA test 39, 138
Encephalitis 25
Endocarditis 20
Endometritis 76, 80
Endovascular infections 20
Enrichment
culture media **151**
stool specimens **41–42**, **146**
Enteric bacteria, colony morphology **48**
Enteric infections (*see also* Stool specimens) **37–38**
Enterobacter
agglomerans, *see Pantoea agglomerans*
cloacae 7, 10, 15, 25, 28, **45–46**, 48, 60, **69–71**
spp. 21, 48
Enterobacteriaceae 4, 7, 10, 15, 28, 144–145, 148, 150
colony morphology 48
identification **45–50**
Kirby–Bauer technique 103
meningitis 25
in pharynx 60, 148
sputum cultures 69, 70, 71
stab cultures 15
susceptibility testing **107**, 116
Enterococci 145
Enterococcus faecalis 11, 63, 64
Enterocolitis 20, 38

Epidemiology **107**
Epididymitis 76, 77
Epiglottitis 60
Epstein–Barr virus 126
Equipment
  anaerobic jars **8**, 44
  blood-culture **22–23**
  care of 7
  operating temperature records **9**
  quality control **8–9**
  serological tests **123**
Error sources, diagnostic tests **2**
Erythrocytes 41
Erythromycin 71, 107, 109, 110, 116, 152
Escherichia
  coli 11–12, 17, 88, 91, 145
    bronchopneumonia 67
    colony morphology **48**
    control strains 14, 110
    diarrhoeal disease **38**, 41
    β-glucuronidase test (PGUA) 35–36
    latex agglutination test 138
    meningitis 25, 138
    performance tests 10–11
    in pharynx 60
    Shigella differentiation 50–51, 59
    stool specimens 37–38, 41, 146
    susceptibility testing 120
    urinary tract infection 30, **35–36**, 94
    wound infections 88
  fergusonii 38
  hermanii 38
Essential drugs, WHO list 152
Ethylenediamine tetraacetic acid (EDTA) 95
Exudates, purulent, *see* Purulent exudates; Surgical specimens

Facultative anaerobic bacteria **98**
Faecal specimens, *see* Stool specimens
False negatives, blood cultures 21
False positives, FTA(Abs) test 131–132
Fasciola hepatica 92
Febrile agglutinins tests **133–135**
Females
  genital specimens **79–82**
  urine specimens **31**
Fevers 37, **133–135**
Filter-paper dip-strip method, urine specimens **33–34**
Flexneri polyvalent antiserum 147, 153
Flocculation reactions, serological tests **125**
Flora
  mixed anerobic 4
  normal pharyngeal **60–61**
Flucloxacillin 108
Fluorescence microscopy 123, 131–132
Fluorescent treponemal antibody absorption (FTA-Abs) test 123, 124, **126**, 127, **131–133**
Francisella tularensis 133
Fungaemia **20–21**
Fungi 10
  fungaemia **20–21**
  meningitis **25–28**
  pharyngitis 61
  surgical specimens **92**, 93
Fusobacterium spp. 61
Fusospirochaetal complex 61, 62

Gardnerella vaginalis 76, 79, 80, 82, 149
Gas gangrene 88, 93, 98, 101
Gastroenteritis **37–38**
Gelatinase 11, 48
Generic names, antimicrobials 108
Genital herpes 76, 82
Genital specimens **77**, **79–82**
Genital ulcers **82–5**
Gentamicin 107, 109, 110, 116, 152
Ghost cells 41
Giemsa stain 85
Glassware
  equipment 8
  serological tests **123–124**
Glomerulonephritis 61, 135
Glucose 27, 46, 52, 63
Glucuronic acid 35
β-Glucuronidase test, *Escherichia coli* 13, **35–36**, 146, 151
Gluteal abscesses 92
Glycerol, long-term preservation 14
Gold immunoassay 138
Gonococcal antimicrobial supplement 151
Gonococcal pharyngitis **61**
Gonococcal urethritis 77
Gonococci, culture preservation 16
Gonococcus, *see Neisseria gonorrhoeae*
Gonorrhoea **61**, 78, 81
Gram-negative organisms 10, 20
  Bacillus anthracis 96
  CSF cultures 27
  meningitis 28, 29
  piperacillin 116
  respiratory tract infection 61
  sputum specimens 69
  stool cultures **45**, **47**
  surgical specimens 87, 91, 96
  urine cultures 34
Gram-positive organisms 10, 20, 27–28, 91
  CSF cultures 27
  sputum specimens 69, 70
  stool cultures **46–47**
  surgical specimens 91, 95
  urine cultures 34
Gram-stained smears (*see also* Gram staining) 64
  bacterial vaginosis **81**
  CSF specimens **26–27**
  sputum specimens **69**
  surgical specimens **91**, 92, 93
  urine specimens 32

Gram staining (*see also* Gram-stained smears) 13, 26, 31, 77, 152
  anaerobes 100, 101
Granulocytopenia 61
Granuloma inguinale (donovanosis) 76, 82, 83, 85
Growth factors 64, 100
Gunshot wounds 88

H factor antisera 147, 153
Haemagglutination tests, stool cultures **53–54**
Haemin 85, 100
Haemolysin 23, 63, 135
Haemolysis
  alpha 11, 28, 50, 70
  antibiotic susceptibility 29
  beta 11, 28, 63, 64
  performance testing 10–11
  reverse CAMP test 101
β-Haemolytic streptococci 61, **63–64**
  grouping kit 148
Haemophilus
  ducreyi 76, 82, 83, 85, 149
  influenzae 10–11, 25, 28, 144, 147, 148
    agglutinating antisera 145, **147**, **153**
    latex agglutination test 138
    lower respiratory tract infections 66, 67, 69, 70, 71
    surgical specimens 93
    susceptibility tests 106
  parainfluenzae 10, 11
  spp. 16, 60, 63, 126
HBV, *see* Hepatitis B virus
HEA, *see* Hektoen enteric agar
Heart infusion agar 42, 48
Hektoen enteric agar (HEA) 42, 48
Hepatitis B virus (HBV) 76
Heroin 127
Herpes, genital 82
Herpesvirus, human (HSV) 76, 77, 80, 82
Heteroresistance 97
Histoplasma capsulatum 92
HIV, *see* Human immunodeficiency virus
Hospital-acquired infection, *see* Nosocomial infection
Hot-air ovens, quality control **8**
HPV, *see* Human papilloma virus
HSV, *see* Herpesvirus; Human herpesvirus
Human herpesvirus 76, 77, 80, 82
Human immunodeficiency virus (HIV) 20, 37, 61, 76, 83
Human papilloma virus (HPV) 76
Hydrogen generator 44, 150, 152
Hydrogen peroxide 44, 152
Hydrogen sulfide 46, 51
Hyphae, fungal 92

Iatrogenic infection 20
Identification (*see also* Interpretation)
  anaerobic bacteria **101–102**
  Bacillus anthracis 93, **96–97**
  Bacillus fragilis **101**
  Clostridium perfringens **101–102**
  Corynebacterium diphtheriae **64–65**
  CSF pathogens **28–29**
  Neisseria gonorrhoeae **79**
  Pasteurella multocida 93, **96**
  Peptostreptococcus spp. **102**
  Salmonella spp. **44–46**, **50–51**
  sputum pathogens **70**
  Staphylococcus spp. **94–96**
  stool pathogens **44–59**
  Streptococcus pyogenes **63**
  surgical specimen pathogens **93–97**
  urine pathogens **32–36**
  Vibrio cholerae **47–48**, **52–53**
  Yersinia enterocolitica **47**, **52–53**, **59**
IFA, *see* Indirect fluorescent antibody test
Immunocompromised patients 20, 60, 82
  diarrhoea 37
  oral candidiasis 61
Immunofluorescence assay 77
Incubation
  anaerobic specimens **99–102**
  blood cultures **23**
  Campylobacter spp. **43–44**
  Clostridium difficile 43
  Kirby–Bauer method 114
  stool specimens **43–45**
  susceptibility tests **118**
  Vibrio cholerae 43
  Yersina spp. 43
Incubators, quality control **8**
India ink 145, 152
Indirect fluorescent antibody (IFA) test 126
Indole 46, 51
Indole test 96
Infants, urine specimens **31**
Infertility 76, 80
Influenza 67
Inhibitors, media/reagent use **151**
Inoculation
  anaerobic specimens **100–101**
  stool specimens **41–43**
  surgical specimens **92–93**
  susceptibility testing **111–114**, 118, **120–121**
  urine specimens 33–34
Inspection, equipment **8**
Intensive-care departments, infections 67
Intermediate susceptibility 105
Interpretation
  cervicovaginal cultures **81**
  Mycobacterium tuberculosis cultures **74**
  sputum cultures **69–70**
  test results 3
  urethral cultures **78**
  urine cultures **34–35**
Iodine 21
Isolation, enteric pathogens **42–44**

Isolation media 43, **144–149**
IsoVitaleX 78, 149, 151

Jars
  anaerobic **8**, 44
  candle 43, 69, 100

Kanamycin 100, 109, 152
Kirby–Bauer disc-diffusion technique (modified) 13, 71, 97
  interpretative chart **116**
  susceptibility testing 103, **104–105**, **109–119**
Kits, diagnostic **151**
Klebsiella
  pneumoniae 10–11, 13, 17, 67, 148
  spp. 48, 60, 91
Kligler's iron agar (KIA) 11, 23, 51, 54
  priority grading 146, 147, 150
  stool specimens **44–49**
Kovacs reagent for indole 46, 146, 147, 152

Laboratories
  appropriate resources use **39–40**
  operations manual 6
Lacerations, infected **89–90**
β-Lactamase production 71, 79, 85, 97, 106, 108
  test reagent 144–145
Lactobacilli, vaginal 80
Lactose 44–45, 46
Lactose non-fermenting bacteria 44–45
Latex agglutination tests 63, **135–139**
Lecithinase 42, 50
Legionella pneumophila 66, 126
Legionnaire disease 66
Leigh & Williams method, urine cultures 33
Leprosy 127
Leptospirosis 20
Leukocyte esterase 32
Leukocytes
  CSF specimens 25–27
  polymorphonuclear 78
  stool specimens 41
  urine specimens 32
Lipase 42, 50
Listeria monocytogenes 21, 80, 144
  meningitis 25, 28, 29
Liver abscesses 92
Löffler coagulated serum 64, 148, 150
Loop technique, calibrated 32, 33
Löwenstein–Jensen medium 28, 67, 72, 93, 145, 148, 150
Lower respiratory tract infections **66–75**
  common **66–68**
  culture/interpretation **69–70**, **72–74**
  diagnostic reagents **149**
  expected pathogens **148**
  media **148–149**
  specimen collection/processing **68–69**
  susceptibility testing **70–71**
Lumbar puncture 25
Lung abscesses **66–67**
Lyme disease 132
Lymph nodes
  spontaneous drainage **90**
  surgical specimens **87**
Lymphogranuloma venereum 76
Lyophilization 14
Lysine 46, 151
Lysine decarboxylase 46, 152
Lysine decarboxylase broth 146, 147

MacConkey agar 11
  anaerobes 100
  CSF cultures 27, 28
  priority grading 144–148, 150
  stool cultures 42, 47–48, 50
  surgical cultures 92, 93
  urine cultures 33–34
Macroscopic evaluation
  CSF specimens **26**
  sputum specimens **68–69**
  surgical specimens **90–91**
Maintenance
  equipment **8**, **123**
  stock cultures **14–16**
Malaria 127
Males
  urethritis **77–79**
  urine specimens **31**
Malonate broth 11
Maltose 64
Mannitol 51–52, 70
Mannitol salt agar 11, 23, 70, 93, 150, 151
Manuals, laboratory operations **6–7**
Mastitis 94
Materials
  ASO tests **136**
  febrile agglutinins test 134
  FTA-Abs test **132**
  RPR test **130**
  serological tests **123–124**
  VDRL test **127**
Measles 67
Media (*see also* Culture media) **7–12**, 42, 142
  anaerobic specimens **99–101**
  blood specimens **22–23**, **144**
  Corynebacterium diphtheriae culture **64**
  CSF specimens **27–28**, **145**
  essential **141–53**
  exudates/pus 92–93, **150**
  lower respiratory tract specimens 69–70, **148–149**
  Mycobacterium tuberculosis culture 74
  Neisseria gonorrhoeae culture 78
  priority gradings 143, **150–151**
  quality control **10–12**
  sputum specimens 67, **69–71**
  stool specimens 41–42, **146–147**
  storage **10**
  Streptococcus pyogenes culture **63**

surgical specimens **92–93**
susceptibility testing **110–111**
upper respiratory tract specimens **64**
urine specimens 33, **145–146**
urogenital specimens **149**
Medusa head 96
Mellibiosa 53
Menadione 100
Meningitis **25–28**, 86
anthrax 96
Escherichia coli 138
neonates 21, 25
Pasteurella multocida 96
pathogens **138**
viral 76
Meningococcus, *see Neisseria meningitidis*
Metacillin, resistance to 17, 62, 97, 108
Methyl red/Voges Prosgauer 11
Methylene blue 8, 41, 64, 77
Meticillin-resistant *Staphylococcus aureus* (MRSA) 17, 62
Metronidazole 102
MIC, *see* Minimal inhibitory concentration
Microaerophilic bacteria 98
Microscopes
maintenance 123
quality control **8**
Microscopic examination
cervicovaginal specimens **81**
CSF specimens **26–28**
genital ulcer specimens **83–85**
pharyngeal specimens **62**
respiratory tract infections **62**
sputum specimens **69**
STD specimens 78, **81**, **83–85**
surgical specimens 86, **91–92**
Treponema pallidum **83–85**
urethral specimens **78**
Microscopy, dark field 50, 82, 83
MIL, *see* Motility–indole–lysine medium
MIU, *see* Motility-indole-urease medium
Mineral oil 15
Minimal inhibitory concentration (MIC) 5, 71, 104–105
Minocycline 108
Mobiluncus spp. 76, 79–80
Monitoring, equipment **8**
Mononucleosis, infectious 126
Moraxella catarrhalis 10, 60, 66, 69, 70, 71, 147, 148
Morganella 51
Morphology, common enteric bacteria colonies **48**
Motility–indole–lysine medium (MIL) 44, **45–46**, 47
Motility–indole–urease medium (MIU) 146–147, 150
MRSA, *see* Meticillin-resistant *Staphylococcus aureus*
MTM, *see* Thayer–Martin medium
Mueller–Hinton agar 11, 14, 20, 24, 54, 71, **110–111**, 120, 150
Mueller–Hinton chocolate horse blood agar 149, 150
Mycetoma 90–92
Mycobacteria, HIV/AIDS 37, 87, 92, 93, 126, 150
Mycobacterium
bovis 74
chelonei 92
fortuitum 92, 93
marinum 92
tuberculosis 25, 66, **72–75**, 87, 92, 145, 148, 150
ulcerans 92, 150
Mycoplasma
hominis 76
pneumoniae 66, 122
Mycoses, lymph nodes 87

N-acetyl-L-cysteine-sodium hydroxide (NALC) 72, **73**
Naegleria fowleri 26
Nafcillin 108
NALC, *see N*-acetyl-L-cysteine-sodium hydroxide
Nalidixic acid 107, 110, 116, 152
Nasopharyngitis 60
Necrotizing ulcerative pharyngitis (Vincent angina) **61**, 62
Needles, serological tests 124
Neisseria
catarrhalis, *see Moraxella catarrhalis*
gonorrhoeae 10, 12, **76**, 77, 79–80, 81, 149
culture **78–79**, **82**
identification **79**
susceptibility testing **79**, 106
meningitidis 10, 12, 21, 25, 28, 29, 144–145, 147
agglutinating antisera 144–145, **147**, **153**
carriers 62
latex agglutination test **138**
susceptibility 106
Neomycin 100
Neonatal infection 80, 82
Neonatal meningitis 21, 25
Netilmycin 109
New York City medium 78, 149
NGU, *see* Nongonococcal urethritis
Nitrate broth 11
Nitrate reduction tests 32, 64, 96
Nitrocefin test 79, 149, 151
Nitrofurantoin 107, 109, 110, 116, 152
o-Nitrophenyl-β-D-galactopyranoside (ONPG) test 13, 51, 146, 147, 151
p-Nitrophenyl-β-D-glucopyranosiduronic acid (PGUA) test 13, 35–36, 151
Nocardia asteroides 93
Nongonococcal urethritis (NGU) 77, 78
Nonproprietary names, drugs 108
Non-trepanomal tests 126–131
Non-tuberculous acid-fast bacilli 92

Norfloxacin 107, 110, 116
Normal flora, pharynx **60–61**
Nosocomial infections 21, 67, **88–89**
Novobiocin 146, 151
Nystatin 78, 151

O antiserum polyvalent 147, 153
Obligate bacteria, aerobic/anaerobic **98**
Oleandomycin 109
ONPG, *see o*-Nitrophenyl-β-D-galactopyranoside test
Operating temperature, equipment 9
Operations manuals **6–7**
Opthalmia neonatorum 80
Optochin discs 13, 23, 28, 69, 70, 144, 145, 148, 149, 151
Oral candidiasis 61–62
Oral surgery 89
Ordering procedures, dehydrated media **7–8**
Ornithine 11, 28, 51
Ornithine decarboxylase 51–52
Otitis media 60, 76
Ovens, hot-air **8**
Oxacillin 71, 97, 107, 108, 110, 116
Oxidase reaction 79, 96
Oxidase reagents 13, 51, 144–153
Oxidase test, stool specimens **49**
Oxygen, bacteria requirement **98**
Oxytetracycline 108

Pantoea agglomerans 21
Paragonimus westermani 92
Parasitic infection, surgical specimens **92**
Pasteurella multocida 88, 150
  identification 93, **96**
Pathogens 143
  blood **144**
  CSF **144–145**
  enteric disease **37–39**, 42
  lower respiratory tract **148**
  pus/exudates **149–150**
  STDs **149**
  stool **146**
  upper respiratory tract **147–148**
  urine **145**
Pelvic inflammatory disease (PID) 76, 80
Penicillins, susceptibility testing 108
Peptone urea broth 147, 150
Peptone water 11, 51
Peptostreptococcus spp. 67, 98–99, **102**
Performance tests
  culture media **11–12**
  prepared media **10**
  stains/reagents 12, **13**
Pericardial sac, exudates 87
Peritoneal cavity, purulent exudate 87, **90**
Petroff procedure, *see* Sodium hydroxide procedure
PGUA, *see p*-Nitrophenyl-β-D-glucopyranosiduronic acid test
Pharyngitis **60–65**, 76
Pharynx, normal flora **60–61**
Phenol red broth 147, 151
Phenylalanine 11, 51
Phenylalanine deaminase 51
pH testing **10**
Piperacillin 107, 110, 116, 156
Pivampicillin 109
Plasma, *Staphylococcus aureus* coagulase test 95–96, 152
Plesiomonas shigelloides 51, 53, 59, 146
Pleural cavity, exudates 87, **90**
Pleural effusion 66, 67
Pleurisy 66
Pneumococcus, *see Streptococcus pneumoniae*
Pneumonia 61, 66, **67–68**
  atypical 127
  Corynebacterium trachomatis 76, 80
Polymorphonuclear leukocytes 78
Polymyxin B susceptibility test 53, **54**
Polysaccharides 63
Polyvidone iodine 21
Polyvitex 151
Porphyromonas spp. 87, 98
Potassium hydroxide 80, 92
Precipitin reactions, *see* Flocculation reactions
Pregnancy 80, 127, 132
Preservation, stock cultures **14–16**
Prevotella melaninogenica 67, 87
Prevotella spp. 98
Priority gradings, media/reagents **143–152**
Proctitis 76
Propionibacterium acnes 21, 24
Prostatitis 30, 76
Protein A 125
Proteus
  mirabilis 10–12
  spp. 10–12, 21, 48, 51, 117
  vulgaris 133
Providentia spp. 48, 51
Prozone reaction 129, 133
Pseudobacteraemia 21
Pseudomembranous colitis 39
Pseudomonas
  aeruginosa 10–11, 87, 90, 110, 144
    bronchopneumonia 67
    susceptibility tests **107**, 116, 120
  cepacia 17
  pseudomalei, *see Burkholderia pseudomalei*
  spp. 22, 91, 145, 147, 150
    in pharynx 60
    sputum specimens 69, 70
Public health, enteric disease 39
Pulmonary tuberculosis 66, **67–68**
Purple lactose agar 145, 150
Purulent exudates 77, **86–97**, **149–150**
  abscesses 67, **89**
  collection/transport **86–87**, **89**
  culture **89**, **92–93**
  diagnostic reagents/media **150**

expected pathogens **149–150**
pathogen identification **93–97**
wound infections **87–88**
Pus 86, 89, 91, 93
lung abscesses 67
macroscopic evaluation **90–91**
Pyelonephritis 20, 30
Pyocyanin 90
Pyogenic bacteria 68
Pyomyositis 94
PYR, *see* Pyrrolidonyl-β-naphthylamide test
Pyrrolidonyl-β-naphthylamide (PYR) test 64, 151

Quality assessment **4**, **16–18**
Quality assurance **2–18**
external **4**
internal **3–4**
Quality control
definition **3–4**
equipment 7, **8**
internal **6–16**
prepared media **10–12**
serological tests **122–124**
stock strains **10**
susceptibility tests **120–121**
Quality criteria, microbiology **4–6**
Quantitative cultures
serological tests **129**, **131**
urine specimens **32–35**
Quinolones 102

Rabies 88
Rapid diagnostic tests
CSF specimens 145, 151
URT specimens 148
Rapid plasma reagin (RPR) test 123–124, 126, **129–131**
Reagents 142, **150–153**
ASO tests **136**
blood specimens **144**
CSF specimens **145**
febrile agglutinins test 134
FTA-Abs test **132**
Kirby–Bauer method 110–111
lower respiratory tract **149**
miscellaneous diagnostic **152**
performance tests 11–12, **13**
priority gradings **143**
pus/exudates **150**
recommended **150–151**
RPR test **130**
serological tests **124**
stool specimens **147**
susceptibility testing **110–111**
upper respiratory tract **148**
urine specimens **146**
urogenital specimens **149**
VDRL test **127–128**
Reagin 126, 127
Records, equipment operating temperature **9**
Rectal swabs, collection **40**
Reference laboratories, use of **16**
Refrigerators, quality control **8**
Reiter syndrome 76
Reliability, diagnostic tests **2**, **5**, 16
Reproducibility, diagnostic tests **2**, **5**
Resistance
antimicrobial **101–121**
metacillin and related drugs 17, 62, 97, 108
Respiratory tract infections, *see* Lower respiratory tract; Upper respiratory tract
Results, reporting **18**
Reverse CAMP test 28, 64, **101–102**
Rhamnose 51, 53
Rheumatic disease 127
Rheumatic fever 61, 135
Rickettsia spp. 126, 133
Rotavirus, diarrhoea **39**
RPR, *see* Rapid plasma reagin test
Rubella 17, 126

Sabouraud dextrose agar 28, 70, 93, 145, 149–151
Saccharose 64
Safety, *Mycobacterium tuberculosis* cultures **74–75**
Saline 112, 124, 127, 128, 132
Salmonella
arizonae 51
choleraesuis 51
groups C and D 58
non-typhi 144
paratyphi 17, 46, 51, 58, 146
spp. 10–12, 25, **37**, 40, 41, 43, 106, 146
agglutinating antisera 144, **147**, **153**
colony morphology 48
identification **44–46**, **50–51**
nomenclature **54–55**
serological identification **54–58**
typhi 17, 21, 42, 51, 107, 133, 144, 146
typhimurium 10–12
Salmonella–Shigella agar 7, 11, 42–44, 48, 147, 150
Salmonellosis 37, 41
Salpingitis 76
Schaedler broth 92, 144
Screening, urine specimens **32**
Selenite F broth 11, 41, 146, 151
Sensitivity, diagnostic **5–6**, 13, 83
Sensitivity testing, *see* Susceptibility testing
Septicaemia **20**, 96
Sera 122, 131
quality assessment **17–18**
serological tests 124
Serological tests 12–13, **122–139**
Salmonella spp. **54–58**
syphilis **126–133**
Serratia marcescens 10, 21, 51, 91

Sexually transmitted diseases (STDs) **76–85**
causal agents **76–77**
diagnostic reagents/media **149**
expected pathogens **149**
specimen collection/transport **77–78**, **80–81**, **83**
specimen culture **78–79**, **82**, **85**
susceptibility tests **79**
Shigella
boydii 38, 46
dysenteriae 38, 42, 47
flexneri 10–12, 36, 38, 46
sonnei 38, 51
spp. 10–12, 16, 17, 20, **37–38**, 146
agglutinating antisera **147**, **153**
final identification **50–52**
identification **44–47**
latex agglutination test 138
serological identification **59**
stool specimens 41, 42, 43, 46–47, 51
susceptibility tests 107, 109
Shigellosis 41
Shock, septic 20
Silver nitrate 83
Simmons citrate agar 11, 23, 51, 146–147, 151
Sinus tract, spontaneous drainage **90**
Sinusitis 60
Sjögren syndrome 127
Skin disinfection
blood collection **21**
CSF collection 25
Skin infections **87–88**, 92, 94
Slide agglutination tests 28, 63, 138
Slide clumping test 95
Smears, *see* Gram-stained smears
Sodium citrate 73
Sodium hydroxide procedure (Petroff), *Mycobacterium tuberculosis* cultures **72–73**
Sodium metabisulfite–sodium pyruvate 42
Sodium polyanethol sulfonate (SPS) 22, 144, 151
Sonnei polyvalent antiserum 147, 151
Sorbitol 63
Soy casein agar, *see* Tryptic soy agar
Specificity, diagnostic **6**, 13, 83, 122, 126, 132
Specimen collection, *see* Collection
Specimen preparation
blood **22–23**
CSF **26**
Specimen transport, *see* Transport
Spiramycin 109
Spirochaetes 61, 84, 126
SPS, *see* Sodium polyanethol sulfonate
Sputum specimens 72–74
collection **68**
culture/interpretation **69–70**
macroscopic evaluation **68–69**
microscopic examination **69**
pulmonary tuberculosis 67, **67–68**
transport 75
Stab cultures 15, 24, 29
Stab wounds 88
Stains (*see also* Gram staining; Ziehl–Neelsen staining)
Albert 64
Giemsa 85
performance tests 12, **13**
Standardization, disc-diffusion methods 119
Standard strains, quality control 120
Staphylococcus
aureus 10, 12, 21, 80, **94–96**, 144, 148
bronchopneumonia 67
carriers 62
identification 70
in pharynx 60–61
sputum specimens 69
susceptibility testing 120
wound infections 88
epidermidis 20, 21, 24, 60, **94–96**
saprophyticus **94–96**, 145
spp. 11–12, 25, 145
surgical specimens 87, 93, **94–96**
susceptibility testing 97, **107**
vancomycin 116
STDs, *see* Sexually transmitted diseases
Sterility testing, prepared media **10**
Stock cultures 10, **14–16**
Stool specimens **37–59**
anaerobic cultures 100
collection and transport **40**
enrichment and inoculation **41–43**
expected pathogens **146**
laboratory resource use **39–40**
media 42, **146–147**
pathogen identification 7, 42–43, **44–59**
reagents **147**
serological identification 54–59
visual examination **41**
Storage
dehydrated media **7–8**
prepared media **10**
stock cultures **14–16**
Streptococcus
agalactiae 10, 21, 25, 28, 29, 64, 145
groups A and B 61, 63, 126, 135
mitis 10
pneumoniae 10, 21, 25, 66, 67, 69, 70, **71**, 145, 148
antibiotic susceptibility 28
latex agglutination test 138
meningitis 25
upper respiratory tract infections 60, 148
pyogenes 10, 21, 61, 62, **63–64**, 126, 135
carriers 62
culture/identification **63**
pharyngitis **61**
priority grading 144, 148, 150
susceptibility 106

spp. 10, 15, 23, 61, 144
ASO test **135–137**
culture preservation 16
surgical specimens 87, 91, 93, 150
Streptolysin O 135
Streptomycin 109
String test, stool specimens **49–50**
Strips, diagnostic **151**
Stuart transport medium 40, 62, 77, 81, 149, 151
Subcultures, blood samples **23**
Subcutaneous tissue, infections **87**
Sucrose fermentation 48, 51–53
Sulfamethoxazole 109
Sulfonamide 107, 108, 110, 116, 152
Sulfur granules 89
Supplements 151
Surgical specimens **86–87**
collection/transport **89**
culture **92–93**
macroscopic evaluation **90–91**
microscopic examination **91–92**
pathogen identification **93–97**
susceptibility testing **97**
Surveys, quality assessment programmes **17**
Susceptibility testing **13–14**, **103–121**
anaerobic specimens **102**
basic antimicrobials **107–109**
benzylpenicillin 108, 110, 116
CSF cultures **29**
direct vs indirect **117**
general principles **103–105**
indirect 117
Neisseria gonorrhoeae **79**
plate size **118–119**
recommended discs **152**
sputum cultures **70–71**
stool cultures **54**
surgical specimens **97**
throat specimens **65**
treatment guide 106
urine cultures **36**
Syphilis (*see also Trepanoma pallidum*) 76, 82, **83–85**
serological tests 13, 125, **126–133**

Tablets, diagnostic **151**
Talampicillin 109
TCBS, *see* Thiosulfate citrate bile salts sucrose agar
Tellurite agar 42, 48, 64, 148, 151
Tellurite discs 13, 23
Tellurite taurocholate gelatine agar (TTGA) 42, 48
Temperature
equipment operating records 9
susceptibility tests **118**
Terminology, microorganisms 5
Tests
agglutination **56–59**, **125–126**, **135–139**
antimicrobial susceptibility **13–14**, **103–121**
ASO **135–137**
diffusion/dilution **103–104**
febrile agglutinins **133–135**
FTA-Abs 123, 124, **126**, 127, **131–135**
media performance **11–12**
ONPG 13, 51, 146, 147, 151
PGUA 13, 35–36
rapid diagnostic 145, 148, 151
RPR 123–124, 126, **129–131**
serological 12–13, **122–139**
stool specimens **49–50**
Tetanus 88, 98
Tetracycline 71, 107, 108, 110, 116
Thayer–Martin medium (MTM), modified 12, 61, 78, 148, 149
Thiamphenicol 108
Thioglycollate broth 12, 23, 38, 92, 144, 150, 151
Thiosulfate citrate bile salts sucrose (TCBS) agar 12, 42, 47–48, 147, 151
Throat swabs
collection **62**
culture/identification **63–65**
gonococcal pharyngitis 61
Thrombophlebitis 20
Tobramycin 107, 109, 110, 116
Tonsillitis 60, **61**
Toxicity assays 38, 64
Toxigenicity 64
Toxic shock 20, 80
Transport (*see also* Dispatch)
anaerobic specimens **99**
cervicovaginal specimens 81
CSF specimens **25**
respiratory tract specimens 62
sputum specimens 68
STD specimen media 149
stool specimens **40**, 146
surgical specimens **89**
urethral specimens **77–78**
urogenital specimens 81
Transport media 81
Amies 40, 41, 62, 66, 77, 81, 149, 151
Buffered glycerol saline 146, 152
Cary–Blair 40–41, 146, 151
Stuart 40, 62, 77, 81, 149, 151
Treatment, susceptibility tests as guide **106**
Treponema
pallidum 76, 82, **83–85**, **125–126**, 149
vincentii 61
Tributyrin 70, 148–151
Trichomonas vaginalis 77, 81
Trimethoprim 78, 107, 110, 116, 152
Triple sugar iron agar 12, 70, 147
Trisodium phosphate 26, 73
Trypanosomiasis, African 26
Tryptic soy agar (TSA) 15, 63, 150
Tryptic soy broth (TSB) 22, 27, 96, 144, 150, 151
Tubercle bacillus, *see Mycobacterium tuberculosis*

Tuberculosis
pulmonary 66, **67–68**
VDRL test 127
Tuberculous meningitis **27–28**
Turbidity standard
Kirby–Bauer technique 111–112
susceptibility testing **111**
Typhoid fever 20, 37

Ulcerative pharyngitis, necrotizing (Vincent angina) **61**, 62
Ulcus molle, *see* Chancroid
Upper respiratory tract infections **60–65**
carriers **62**
causal agents **61–62**
diagnostic reagents/media **148**
direct microscopy **62**
expected pathogens **147–148**
pathogen culture/identification **63–65**
Urea medium 12, **44–45**, 76, 77
Ureaplasma urealyticum 77
Urease 12, 45, 47, 51, 53
Ureteritis 30
Urethral specimens
collection/transport **77–78**
interpretation **78**
Urethritis
in men **77–79**
in women 80
Urinary tract infection (UTI) 30, 35, 94
Urine **30–36**
culture/interpretation **32–35**, 100
diagnostic reagents/media **145–146**
expected pathogens **145**
screening **32**
specimen collection **30–32**
Urogenital specimens **149**
UTI, *see* Urinary tract infection

Vaginitis 76, 79, **81**
Vaginosis 76, **79–82**
Vancomycin 78, 110, 116, 149, 151, 152
VCN mixture 78, 151
VDRL, *see* Venereal Disease Research Laboratory test
Venepuncture, blood collection **21**
Venereal Disease Research Laboratory (VDRL) test 123–124, 125
syphilis 126, **127–129**
Ventricular puncture 25
V-factor 13, 144–151
Vibrio
cholerae 10, 16, **38**, 146
agglutinating antisera 147, **153**
identification **47–48**, **52–53**
media 41, 42, 43
fluvialis 38, 53
furniss 53
hollisiae 38, 53
mimicus 38, 53
parahaemolyticus 38, **47–49**
vibriostatic compound O:129 disc 147, 151
Vincent angina, *see* Necrotizing ulcerative pharyngitis
Viral diarrhoea 41
Viral meningitis **27**
Vitox 151
Voges–Prosgauer agar 11, 52, 53

Water-baths, quality control **8**, 123
Weil–Felix reaction, rickettsias 133
White blood cells, *see* Leukocytes
Widal test, *Salmonella typhi* 6, 133
Wilkins–Chalgren anaerobe broth 100, 144, 151
Wounds
Clostridium spp. 98
nosocomial infections **88–90**
penetrating **87–90**, 96
Wright test, *Brucella* 133

XLD, *see* Xylose–lysine–deoxycholate agar
XV-factor 13, 85, 144–145, 148–149, 151
Xylose–lysine–deoxycholate (XLD) agar 42–44, 48, 147

Yeasts (*see also* Fungi) 26, 69
Yersinia
enterocolitica 17, 39, 133, 146
colony morphology 48
identification **47**, **52–53**, **59**
media 41, 42, 43
frederiksenii 53
intermedia 53
kristensenii 53
pseudotuberculosis 53

Zephiran–trisodium phosphate procedure **73–74**
Ziehl–Neelsen staining 74, 152
CSF specimens **27**, 28
diagnostic specificity 6
pulmonary tuberculosis 67
sputum specimens 67
surgical specimens **92**, 93
Zone diameters, disc-diffusion susceptibility testing 104–105, **110–111**, **116–119**